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PROTEIN KINASE C MODULATORS: SPECIFICITIES IN HUMAN PLATELETS, AND EFFECTS ON THE EXPRESSION OF NUCLEOSIDE TRANSPORTERS IN CULTURED HUMAN T-LYMPHOBLASTOID CCRF-CEM CELLS

by

© JAMES KWAKU TURKSON

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA
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c/o Mr. Tony T. Turkson
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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Protein Kinase C modulators: specifities in human platelets, and effects on the expression of nucleoside transporters in cultured human T-lymphoblastoid CCRF-CEM** cells, submitted by **James Kwaku Turkson** in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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24 JANUARY 1995

To the Glory of God,

and to my wife, Rita

Abstract

The objective of part I of this project was to investigate non-protein kinase C (PKC)-specific effects of staurosporine, (STA), the most potent of the known PKC inhibitors, on inositol phosphate metabolism in human platelets. Micromolar concentrations of STA caused significant formation of inositol trisphosphate levels in both intact and permeabilized human platelets. In intact platelets, this effect was blocked by neomycin, a phospholipase C inhibitor. STA, however, induced only a weak transient rise in $[Ca^{2+}]_i$ levels but potentiated the thrombin-induced $[Ca^{2+}]_i$ rise in platelets. STA did not induce significant platelet responses, such as aggregation. Studies that employed platelet membranes showed that STA exhibited a stimulatory effect on GTPase activity, suggesting that the PKC inhibitor might activate phospholipase C-phosphatidylinositol 4,5-bisphosphate pathway in human platelets via a G protein.

Following these studies, we also employed STA and other PKC modulators in studies of the activity of nucleoside transporters in cultured, undifferentiated T-lymphoblastoid CCRF-CEM (CEM) cells. The search for effective anticancer agents has intensified and thus, has enhanced the studies of nucleoside analogs as therapeutic agents for cancers. Since nucleoside entry into cells depends on the activity of nucleoside transport (NT) processes, an understanding of the regulation of nucleoside transporter numbers and activity may lead to new ways of improving the therapeutic effectiveness of nucleosides in cancer chemotherapy. The role of PKC in the expression of the *es* (nitrobenzylmercaptopurine riboside, (NBMPR)-sensitive) NT process in CEM cells was examined in part II of this project. Phorbol 12-myristate 13-acetate (PMA), and STA, both inhibited inward fluxes of labeled adenosine (Ado) and reduced the cellular abundance, B_{max}, of NBMPR binding sites without altering the equilibrium dissociation constant, K_D, CEM cells. The decreases in B_{max} of NBMPR binding sites and rate of Ado influx were both time- and concentration-dependent. Each of these agents also reduced the V_{max} of Ado influx without affecting K_m, in CEM cells treated for 16 h with

PMA or STA. Both phorbol 12,13-dibutyrate, and another PKC inhibitor, H7, also reduced the B_{max} for NBMPR binding and decreased Ado influx rates, while the inactive phorbol ester, 4α-phorbol didecanoate, and inhibitors of protein kinase A, cGMP-dependent protein kinase or tyrosine kinase, did not exert observable effects. These results suggest that PMA and STA exert their effect via PKC. The results also suggest that PKC down-regulation by phorbol ester or STA-mediated PKC inhibition, underlies the decrease in NBMPR binding site abundance and Ado influx rate. Assay for PKC in CEM cells treated for 16 h with PMA showed significant reduction in PKC activity in both membrane- and cytosolic fractions, confirming that treatment with PMA induced the down-regulation of PKC under these conditions. Both STA and PMA also significantly caused a decline in cell numbers following 16 h drug-treatments of CEM cells consistent with other reports that these agents induce differentiation. Studies with inhibitors of protein phosphatases 1 and 2A, okadaic acid (OKA) and calyculin A (CLA), showed that these agents alone did not alter the B_{max} for NBMPR binding or Ado influx rates. However, both OKA and CLA blocked the STA-induced decrease in the B_{max} of NBMPR binding and CLA also abolished reduction in rate of influx of Ado by STA. Thus, the results of this study are consistent with the hypothesis that protein phosphorylation and dephosphorylation processes play a role in the regulation of *es* NT processes in CEM cells. Altogether, results of both parts of this study indicate that at higher than nanomolar concentrations, the PKC inhibitor, STA, caused non-PKC-specific effects in platelets; however, treatment of CEM cells with STA or PMA at nanomolar concentrations, which affect PKC activity, reduced the numbers of high affinity NBMPR binding sites and nucleoside transporter activity in those cells.

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LIST OF ABBREVIATIONS

Ado.	β-D-Adenosine, 9-β-D-ribofuranosyladenine
ATP	Adenosine 5'-triphosphate
^{32}P ATP	γ-^{32}P Adenosine 5'-triphosphate
cAMP	cyclic Adenosine 5'-monophosphate
BIM	Bisindolylmaleimide, GF 109203X
B_{max}	Maximum number of ligand binding sites
BSA	Bovine serum albumin
CCRF-CEM	Undifferentiated T-lymphoblastoid cells (CEM)
CLA	Calyculin A
$[Ca^{2+}]_i$	Cytosolic Ca^{2+} concentration
DAG	1,2-diacylglycerol
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid
FBS	Fetal bovine serum
Gp	Guanine nucleotide binding protein
Gi	Inhibitory guanine nucleotide binding protein
cGMP	cyclic Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine 5'-[γ-thio]triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
H7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride
HA1004	*N*-(2-guanidinoethyl)-5-isoquinlinesulfonamide

	hydrochloride
IC_{50}	Concentration of drug(s) inhibiting the parameter measured by 50%
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
$Ins(1,3,4)P_3$	Inositol 1,3,4-trisphosphate
$InsP_3$	Inositol trisphosphates: $Ins(1,3,4)P_3$ and $Ins(1,4,5)P_3$
kDa	Kilo-Dalton
NBMPR	Nitrobenzylmercaptopurine riboside; 6-[(4-nitrobenzyl)-thio]-9-β-D-ribofuanosylpurine
NT	Nucleoside transport
OKA	Okadaic acid
PDBu	Phorbol 12, 13-dibutyrate
4α-PDD	4α-phorbol didecanoate
PMA	Phorbol 12-myristate 13-acetate, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PP1/PP2A	Protein phosphatase 1/Protein phosphatase 2A
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulfonyl fluoride
RPMI-1640	Roswell Park Memorial Institute Medium 1640
S.D.	Standard deviation
STA	Staurosporine
TCA	Trichloroacetic acid
Tris-HCl	Tris(hydroxymethyl)-aminomethane hydrochloride

V_{max}	Maximum rate of influx of nucleoside under conditions when transporter protein is saturated with nucleoside
$^{3}H_{2}0$	tritiated Water

1. INTRODUCTION

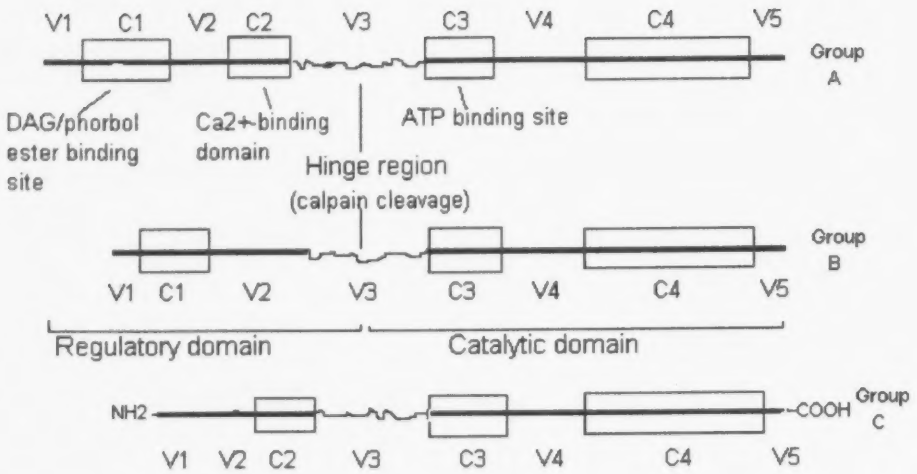
Cellular processes such as secretion, cell proliferation and differentiation, and a host of neuronal responses, are regulated by external signals. Hormones, growth factors, neurotransmitters, and some biologically active, are some of the variety of agents that may act externally to initiate these processes. Among the biochemical pathways that serve as transducing mechanisms for the external agents is receptor-mediated phosphoinositide hydrolysis, which is operative in most cells. This pathway involves protein kinase C (PKC), a seemingly ubiquitous enzyme. There is now a great deal of evidence to demonstrate that PKC may be involved in the control of a number of these biological processes (see 1, 2, 3 for reviews). PKC is a family of serine- and threonine- specific protein kinases which have been identified functionally by common enzymatic properties or by common structural features (1). Biologically, PKC is transiently activated in cells by 1,2-*sn*-diacylglycerols (DAG) which are produced by the action of specific phospholipase C (PLC) (4). Transduction of signals, from Ca^{2+}-mobilizing agonists, across the cell membrane by cell surface receptors involves PLC-mediated hydrolysis of phosphoinositide, phosphatidylinositol 4,5-bisphosphate (PIP_2) with the formation of Ins(1,4,5)P_3, the Ca^{2+}-mobilizing second messenger and DAG (4).

1.1. PKC

1.1.1 PKC Molecular Structure and Heterogeneity

Presently, there are about 12 different isozymes in the family of PKC, which are represented as α, βI, βII, γ, δ, ε, ε', ζ, η, θ, ι, λ and μ (5-9, for review, 3, 10, 11) However, at this time, no sequence data are available for PKC-ι, -λ and -μ (see 9 for review). The existence of different isozymes of PKC may be the reason for the variety of processes that the kinase is said to be involved in.

These isozymes are divided into three groups, based on their primary structure and enzymatic properties (see 5-9, for reviews, 1, 3, 10). The first, group A enzymes called conventional PKCs, include -α, -β (-β1 and -β2), and -γ isozymes and these require calcium, phospholipids, and diacylglycerol for activation. These enzymes were obtained as products of corresponding α, β, or γ genes, initially cloned from brain cDNA libraries of several species (8, 10, 12-16). They are single polypeptides, containing four conserved (C1-C4) and five variable (V1-V5) regions. The C1 region is the phospholipid- and DAG/phorbol ester-binding region containing the pseudosubstrate sequence, -Arg-Lys-Gly-^{25}Ala-Leu-Arg-Gln-Lys-, responsible for maintaining the enzyme in the inactive state in the absence of an activator (17). Substitution of Ala 25 with serine transforms the pseudosubstrate to a substrate. The same region also contains a tandem repeat of a cysteine-rich Zn^{2+} finger motif which confers the phorbol ester binding activity of all PKCs (18); C2 is the calcium binding domain which is highly conserved in the group A PKCs. The ATP binding site sequence, -Gly-X-Gly-X-X-Gly-X16-Lys-, shared by many other kinases, is contained in the C3 region and forms part of the catalytic domain. The second group, or group B enzymes, which consists of PKC-δ, -ε, and -ε', and -η lacks the calcium binding C2 section and as such, purified representatives of these have been found to be insensitive to calcium (1, 3, 6-10, 19). The PKC-θ, expressed predominantly in skeletal muscle, hematopoietic cells and also in platelets (20, 21, 22) shows the highest homology to PKC-δ. It lacks the calcium binding C2 domain, and has been placed in the subfamily of calcium-independent PKC isozymes (20, 21). PKC-μ is also said to belong to second group B isozymes (9). The third group of PKC enzymes consists of PKC-ζ and -λ (8). PKC-ι bears a relationship with PKC-ζ, and therefore has also been placed in this subfamily (see 9 for review). These isozymes are not activated by DAG or phorbol esters, but are stimulated by phosphatidylserine or unsaturated fatty acid (8). All the isozymes of PKC exhibit the basic two functional catalytic and regulatory domains (see diagram of domain structure of PKC below).



DOMAIN STRUCTURE OF PROTEIN KINASE C

DIAGRAM 1

The C-terminal catalytic domain contains sections C3, C4 and V4 (12) with the ATP- and substrate-binding sites. The N-terminal regulatory domain contains C1, C2 and V1, V2, and part of V3. This domain interacts with Ca^{2+}, phospholipid, DAG, or phorbol ester. Also contained in the regulatory domain is the autoinhibitory pseudosubstrate sequence (17, for reviews see 1, 3, 6, 23-25, and other references therein). Agents such as calcium ions, *sn*-DAG, arachidonic acid, and phospholipids such as phosphatidylserine (PS), lysophosphatidylcholine, phosphatidylinositol phosphates, phorbol esters, and bryostatin, are capable of unmasking the catalytic domain by binding to sites on the regulatory domain and relieving the autoinhibition (1, 3, 6, 19, 26-29).

The expression of PKC isozymes is tissue-specific. For example, the PKC-α, -δ and -ζ are expressed in a wide variety of tissues. The γ, η and θ isozymes of PKC are limited in their expression to one or few tissues (11). Furthermore, only a particular isozyme may be present in a cell (11). Also, within a cell, localization of isozymes may

differ, although it has been proposed that cellular localization of the PKC isozymes may be a dynamic process which could be influenced by the activation state of the cell (see 9 for review). Such observation has been made in fibroblasts where PKC-α, but not the -ε and -ζ isozymes were translocated from the cytosol to the nucleus when cells were stimulated with α-thrombin (see 9 for review).

1.1.2. PKC autophosphorylation

PKC has been found to have the ability to phosphorylate itself (30). Both the catalytic and regulatory domains are known to be autophosphorylated (31, 32). The process of autophosphorylation has been shown to alter the affinity of the enzyme for phorbol esters, to increase its sensitivity to Ca^{2+}, and to increase its rate of histone H1 phosphorylation (see 24 for review). The association of PKC with the membrane is also found to be dependent on autophosphorylation (33, 34). In PKC autophosphorylation, a single polypeptide chain phosphorylates itself via an intrapeptide mechanism. There are six major sites of PKC autophosphorylation that have been identified suggesting that multiple phosphorylation could be a mechanism of producing a number of protein kinases with slightly altered specificities (35). Modified residues found so far are in the amino terminal peptide, the carboxyl terminal tail and the hinge section between the regulator and catalytic domains (35). Phosphorylation at the hinge section is rather close to the site of cleavage by calpain and is therefore likely to alter the susceptibility of PKC to proteolysis (see 24 for review).

1.1.3. Activation of PKC

In cells, PKC may exist in a cytosolic pool, a membrane-bound form that solubilizes with chelators, and a membrane-bound (or membrane-inserted) form that requires detergent for solubilization (24, 36, 37). The membrane-inserted form has been

found to be endogenously active and no further increase in activity could be elicited by addition of Ca^{2+} or phorbol ester (see 24 for review).

Activation of PKC involves the movement of cytosolic PKC to the plasma membrane, the phenomenon referred to as translocation. PKC thus associated with the membrane is then in contact with phosphatidylserine (a membrane phospholipid), and in the presence of calcium and DAG, the kinase becomes activated (see 7, 37, 38 for reviews). It is said that PKC-phospholipid interaction is required for the activation of PKC, but this interaction does not evoke the kinase activity (see 37 for review). It has been argued that the PKC-phospholipid interaction occurs in the presence or absence of a divalent metal ion; however, in the absence of a divalent metal ion, both the regulatory and catalytic domains of the kinase bind phosphatidylserine and polyphosphoinositides keeping the kinase in the inactive state (see 37 for review). Other agents, including tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), and bryostatin 1, are known to be potent activators of PKC (6, 7, 27-29). Both phorbol esters and bryostatin 1, as well as synthetic diacylglycerols, such as 1-oleoyl-2-acetyl glycerol and 1,2-dioctenyl glycerol, have the ability to activate PKC through binding at the DAG binding site (39-43). It has been shown that both of these groups of compounds can substitute for DAG as PKC activators (39-43). Phorbol esters as well as bryostatin 1 induce longer-lasting PKC activation than the physiological activator, DAG, and bryostatin has been found to act much more rapidly in inducing PKC activation than phorbol esters (28, 29).

1.1.4. Down-regulation of PKC

Prolonged exposure of PKC to nonphysiological activators such as phorbol esters or bryostatin 1 leads to initial activation followed by rapid degradation of the enzyme (6, 28, 29, 44). The degradation phenomenon is termed down-regulation. During this event, there is translocation of PKC from the cytosol to the membrane, with consequent degradation

upon persistent activation. The degradation is achieved by the action of proteolytic enzymes associated with the membrane (2, 3). *In vitro*, PKC can be degraded by trypsin or calpain into two fragments containing a catalytic and a regulatory domain (37). The physiological importance of PKC down-regulation is uncertain, although it may serve as a means to curtail persistent consequences of activated PKC.

1.2. INOSITOL PHOSPHATES SIGNAL TRANSDUCTION PATHWAY IN PLATELETS

1.2.1. The PKC-PLC-PIP_2 Pathway

The signal transduction pathway by which PKC becomes activated biologically is well characterized in some cell systems. In platelets and neutrophils, there is a substantial volume of data that relates the responses of these cells to activation of the PLC-PKC pathway (1-4, 44-46). This pathway is also utilized by other Ca^{2+}-mobilizing agonists (4). Upon stimulation by suitable agonists, such as thrombin, collagen, etc., platelets undergo observable responses that begin with shape change (from discoid to a more rounded form), primary aggregation which is reversible, and terminating in an irreversible, secondary aggregation (47, 48). The response to platelet agonists is triggered following the binding of the agonists to their respective surface membrane receptors. These receptors are imbedded in the plasma membrane by seven putative transmembrane segments (47). This induces the hydrolysis by PLC of phosphatidylinositol 4,5-bisphosphate (PIP_2), with the formation of DAG and inositol 1,4,5-trisphosphate (IP_3) (4, 49). Coupling the agonist receptor to PLC is a guanine nucleotide-binding protein, B protein (G_p) (4). Direct protein isolation, immunological characterization and molecular cloning have revealed at least four types of PLC: PLC-α, PLC-β, PLC-γ, and PLC-δ, all of which are single polypeptides (50, 51). Each type contains more than one subtype, with each subtype designated by arabic numerals following the Greek letters, for example PLC-β1 and PLC-β2, etc. (50, 51). In platelets, evidence has accumulated supporting the fact

that one or more guanine nucleotide-binding proteins, regulate the PLC activity although in some tissues, some isotypes of PLC may not be regulated by G proteins (see 49, 52 for reviews). For example, while it is established all isotypes PLC-β, (that is PLC-β1-4) are regulated by G proteins others like PLC-γ, are not (see 52 for review).

The guanine nucleotide-binding proteins also consist of three subunits, α, β, and γ, which form a heterotrimer (see 52, 53 for reviews). It is generally believed that the activated α- subunit binds GTP forming the G_α-GTP, and interacts with the particular effector (in this case PLC) upon agonist stimulation of the receptor. However, evidence has accumulated to indicate that β and γ subunits, which form hydrophobic dimers, may also play a major part in signal transduction by activating some effectors in the same way as G_α-GTP does (see 53, 54 for reviews, 55). Apart from regulating the α functions and anchoring the α to membranes, the βγ dimer stimulates phospholipase A2 (56, 57) and is capable of binding Ca^{2+}-calmodulin (49, 54, 58). Several distinct G_α subunit genes have been identified, of which at least 7 distinct isoforms are present in platelets- αs, αi1, αi2, αi3, αq, αz, and α13 (58, 59, 60). The G proteins regulating PLC may be sensitive or insensitive to pertussis toxin (47, 49, see 58, 61 for reviews). Some platelet agonists such as thrombin and PAF act on receptors that are coupled to PLC via a pertussis toxin-sensitive inhibitory G protein, G_i (49). There are at least four different Gβ subunits, $G_{\beta 1}$... $G_{\beta 4}$, and seven different G_γ subunits, $G_{\gamma 1}$, $G_{\gamma 2}$, ... $G_{\gamma 7}$ (see 53 for review).

Of the two second messengers produced in the PLC-PIP_2 transduction pathway, Ins(1,4,5)P_3 diffuses into the cytoplasm and mobilizes Ca^{2+} from non-mitochondrial stores, such as endoplasmic reticulum (45, 46), while DAG remains in the membrane, and in the presence of phosphatidylserine, activates PKC (49, 62). Calcium has been shown to play a prominent role in platelet responses by activating platelet response elements such as PKC, phospholipases, cytoskeletal proteins involved in actin polymerization, that depend on Ca^{2+}, or in combination with calmodulin to activate myosin light chain kinase (see 49 for review). There is substantial evidence to suggest that PKC may act with Ca^{2+} to

produce cellular responses. For example, in studying platelet responses, such as shape change, secretion and aggregation, Watson and others (1988) observed a reinforcement between PKC-mediated and Ca^{2+}-induced effects that indicated a synergism between the effects mediated by the two arms of the signal transduction pathway (4, see also 45, 46 for review). Various groups have shown, however, that DAG/PKC pathway is important in the secondary phase of platelet activation, that is induction of secretion and secondary aggregation (63-66). The accumulation of the levels of DAG in response to agonist stimulation was found to be delayed, consistent with the supposition of activated PKC-mediated platelet secretion and secondary, irreversible aggregation (66).

There are reports of a correlation between polyphosphoinositide turnover/Ins (1,4,5,)P_3 formation and platelet responses such as shape change, aggregation and secretion (61), although in some cases platelet responses have been observed without the formation of inositol phosphates (see 61 for review).

1.2.2. Interaction between PLC and PIP_2

There is evidence to suggest that in resting platelets and neutrophils, some actin binding proteins, particularly profilin, may bind PIP_2 tightly. It has been demonstrated *in vitro* that profilin binds specifically four or five molecules of PIP_2 while the profilin-actin complex dissociates (67-70). This results in shielding by profilin of PIP_2 from hydrolytic action of PLC, and this has been specifically observed for the interaction between PIP_2-PLC-γ in platelets (71). It has been demonstrated, too, that a significant amount of profilin is in close association with both the internal leaflet of the plasma membrane as well as the external surfaces of intracellular membranes in human leukocytes and platelets (72). It has been proposed that such locations for profilin may reflect the direct binding of profilin to PIP_2 and phosphatidylinositol 4-phosphate (72).

1.2.3. RESEARCH OBJECTIVES

Non-PKC specific effects of staurosporine on the PKC-PLC-PIP_2 transduction pathway in human platelets

Investigations into the role of PKC in cellular processes have involved the use of various PKC activators, such as phorbol esters, bryostatins, and as well the use of PKC inhibitors like H7, STA, K252a, auranofin, to mention but a few (see 73, and 74 for reviews, 75).

Phorbol esters are natural products from *Croton tiglium* (source of croton oil) and from plants of *Euphorbiaceae* family (76). They are very potent co-carcinogens, or tumor promoters, but, by themselves, are not mutagenic or carcinogenic (77-79). However, chronic administration of tumor promoters after an animal has been exposed to a carcinogen at minimal effective dose, would result in the appearance of skin tumors. Until recently, PKC was the only known receptor for phorbol esters (10, see 12 for reviews). However, it has been reported quite recently that neuronal chimaerin, in the brain, binds phorbol esters with high affinity, stereospecificity, and requires phospholipids for its action as a p21*rac*-GTPase activating protein (80, see 8 for review). The activity of this protein, however, remains to be elucidated to provide a better understanding of the role it plays in inositol phosphates signal transduction. H7 is an isoquinoline which was one of the first PKC inhibitors shown to bind to the ATP-binding sites on the catalytic domain of PKC (81, 82, see also 6 for review). STA and K252a are both microbial products obtained from *Streptomyces* sp. (83, 84) and *Nocardiopsis* sp. (85-87), respectively. These inhibitors, similar to H7, bind to PKC at the ATP-binding site on the catalytic domain (see 74 for review) and therefore prevent ATP from binding. As a result, these inhibitors are nonspecific and do inhibit other kinases (see 74 for review).

Since PKC inhibitors are not specific, results from studies involving their use might have included non-PKC specific effects. For instance, Wong and others found that STA and auranofin, another PKC inhibitor, elevated cytosolic free Ca^{2+} levels in resting human

neutrophils (75, 88). Also, except in situations where studies to rule out STA-mediated inhibition of other kinases were conducted, the effect of STA measured might have been the result of inhibition of other kinases such as PKA or tyrosine kinase since the IC_{50} values are similar. (IC_{50} values for inhibition of PKA, tyrosine kinase or PKC by STA are 8 nM, 6 nM and 3 nM respectively) (74).

Early studies of the use of STA in investigating the role of PKC in the polyphosphoinositide signal transduction pathway employed high concentrations of this inhibitor, and yet nonspecific effects of STA were not considered, although significant at the high concentrations used. In some of these papers, concentrations of STA as high as 10 μM were used (4, 89-91, to cite but a few). The studies reported by Wong and others (88) showed that STA raises basal levels of cytosolic Ca^{2+} in human neutrophils. Following that, attention was directed toward nonspecific effects of STA in human platelets. Therefore in this project, the focus was to examine and identify some of the nonspecific effects of STA in polyphosphoinositide signal transduction, and the possible ways by which this drug, at submicromolar concentrations (normally used to inhibit PKC), could mediate these nonspecific effects in human platelets. A literature search showed that a significant amount of work has been done in the area of polyphosphoinositide signal transduction in platelets and therefore this cell type was chosen for part I of the thesis project. The effect of STA on basal cytosolic Ca^{2+} levels, on the hydrolysis of PIP_2 (the source of IP_3) by PLC, and the influence of STA on the activity of the G protein that couples the agonist receptor to PLC are, among others, the main subject of part I of this project.

1.3. THE EFFECTS OF PKC ON THE ACTIVITY OF NUCLEOSIDE TRANSPORTER AND ON PROLIFERATION OF CULTURED TUMOR CELLS

1.3.1. PKC and membrane transport

It has been observed that changes in some membrane transport processes may be mediated by PKC. For example, transporter-mediated glucose permeation (92) and nucleoside transport (NT) (93-95), have been reported to be altered by phorbol ester treatments. Also, treatment of cells with STA has been reported to result in changes in NT expression (95).

1.3.1.1. Role of PKC on the activity of glucose transporter

In most mammalian cells, the uptake of glucose proceeds by facilitated diffusion which is driven by the concentration gradient existing across the plasma membrane (see 96, 97 for review). However, there are also secondary-active, Na^+-linked transporters present in small intestine and kidney (see 97 for review). Glucose transporters comprise a family of at least 7 homologous glycoproteins, designated GLUTS, with similar kinetic properties. PKC has been implicated in the activity of these transporters.

Results of studies that implicate PKC in the regulation of glucose transport include the reported PKC-mediated phosphorylation of the purified human erythrocyte glucose transporter protein (50 - 60 kDa) *in vitro*, and of an 80 kDa protein following incubation of ^{32}P-labeled human erythrocytes with PMA (92). Also reported was the observation that stimulation of PKC by phorbol esters led to an increase in glucose transport as well as in GLUT1 gene transcription in quiescent mouse fibroblasts, BALB/c 3T3, and other cell types (98-100). Witters and others (92) inferred that the phorbol ester-stimulated glucose uptake could in part result from PKC-mediated phosphorylation of glucose transporter polypeptides. The response of glucose uptake to phorbol esters may be complex, and vary in response time and requirement for protein synthesis (99, 100). It has been reported that

activation of PKC by phorbol esters resulted in a reduction in degradation of GLUT1 mRNA in preadipocytes (see 96 for review).

1.3.1.2. Nucleoside transport

The transport of nucleosides across the plasma membranes of animal cells involves at least six distinct carriers (see 101, 102, for reviews, and 103). These are broadly classified into two categories, the equilibrative, facilitated diffusion carriers, and the concentrative, Na^+-dependent carriers. Two equilibrative carriers are known, one designated *es*, is of high sensitivity to the nucleoside transport inhibitor, nitrobenzylmercaptopurine riboside, NBMPR (nitrobenzylthioinosine), and another of low sensitivity to NBMPR, designated *ei* (104-106). The *es* and *ei* transporters have similar kinetic properties and broad permeant specificity (see 102 for review). The equilibrative carriers mediate both the influx and efflux movements of nucleosides. The other broad class is the concentrative, Na^+-dependent carriers. Exploring the substrate specificity of the Na^+-linked transporters led to further subclassification into at least three activities: a Na^+-dependent transporter insensitive to NBMPR, that accepts formycin B (a poorly metabolized analog of inosine) , but not thymidine as a permeant (designated *cif*); a Na^+-dependent transporter insensitive to NBMPR, that accepts thymidine (a physiological pyrimidine nucleoside), but not formycin B, as a permeant (*cit*) (104-106, see 102 for review); and a Na^+-dependent transporter insensitive to NBMPR, which accepts a broad range of purine and pyrimidine nucleosides as permeants (*cib*) (104-107, see 102 for review). Quite recently, Paterson and others (103) have reported the presence of NBMPR-sensitive Na^+-dependent transport of nucleoside analogs in acute myelogenous leukemia (AML) and chronic lymphocytic leukemia (CLL) cells and this they designated *cs* (*c*oncentrative, NBMPR-*s*ensitive) nucleoside transport. Both the two equilibrative transporters as well as the Na^+-dependent active transporters have been identified in normal and in tumor cells (see 101, and 102 for reviews, 103).

There are reports of correlations between the proliferation rate of cells and the abundance of NBMPR binding sites (nucleoside transporters) in cultured cells as well as in fresh human tumor cells (108-111). Rapidly proliferating cells have a higher abundance of NBMPR binding sites as well as higher nucleoside transporter activity than non-proliferating cells (108-111).This correlation has been reported in fresh human leukemia and lymphoma cells (108-111) and in fresh human thymocytes (111). Proliferating human leukemic blast cells possessed a higher density of NBMPR binding sites than their mature nonproliferating differentiated counterparts (109, 110). In cultured cells, the abundance of NBMPR binding sites and rates of nucleoside uptake increased substantially as synchronized HeLa cells progressed from G1 through S phases of the cell cycle (112). Also reported was the observation that sheep red cells lost nucleoside transport activity and NBMPR binding sites during transition from reticulocyte to the erythrocyte stages (113). When cultured cells are induced to differentiate, changes in NT activity and in NBMPR binding site numbers are observed as well. Cultured HL-60 cells induced to differentiate along the myeloid pathway progressively lost high affinity NBMPR binding sites and nucleoside transport activity (114). Other observations show that changes in nucleoside transport activity occurred when cells were induced to differentiate (93, 94, see next section).

PKC and nucleoside transport

Changes in nucleoside transporter activity have been observed during prolonged treatment of cells with phorbol esters (92-94), and also during treatment with STA (95). Reports in the literature about the role of PKC in nucleoside transport have been inconsistent. In some cases, observed changes in NT activity, as a result of prolonged PMA-treatment of cells (a condition that would down-regulate PKC), were attributed to PKC activation. For example, the decline in facilitated diffusion nucleoside transporter activity and induction of Na^+-dependent uridine uptake that resulted from 48 h treatment

of HL-60 cells with PMA was attributed to PKC activation (93). While prolonged treatment of cells with PMA caused a decline in facilitated nucleoside transporter activity in HL-60 cells (93), a 10 min treatment of chromaffin cells with PMA or PDBu caused a decline in B_{max} of NBMPR binding sites, as well as in the rate of Ado influx (95). Furthermore, treatment of chromaffin cells with STA for 10 min increased high affinity NBMPR binding while PKC down-regulation (a situation which would eliminate PKC activity and thus be similar in effect to STA exposure) did not show changes in B_{max} of NBMPR binding sites (95). In other studies, exposure of MOLT-4 human T-lymphoblastoid cells to PMA for 4 days produced a decrease in cytosine arabinoside (araC) transport as well as transport of thymidine across the cell membrane (94), suggesting that PKC down-regulation is the cause of the decrease in transporter activity. Based on these findings, deductions about the effects of prolonged incubation of cells with PMA on cellular abundance of NBMPR binding sites and NT activity would be difficult to make, because of the conflicting nature of the results. For instance, in the above studies, there was no effect on one hand (95), and a reduction in nucleoside transporter activity on the other, following prolonged incubation of cells with PMA (93, 94). The increase in B_{max} of NBMPR binding sites following inhibition of PKC with STA also conflicts with the observation that down-regulation of PKC had no effect on nucleoside transporter activity (95). This is because in some studies, PKC inhibitors have been used to achieve the same effect as phorbol ester-induced PKC down-regulation, that is, elimination of PKC activity (115-118). Notwithstanding these conflicts, however, the differences in cell type used could also have affected the outcome of the studies. Thus, the existence of such conflicts makes further work in this area necessary.

1.3.2. PKC, cell proliferation and differentiation

Physiologically, cell proliferation is the result of a sequence of events mediated by protein kinases in response to stimulation of growth factor receptors. The control of cell

proliferation and of differentiation is exerted at the gene level. Responses to specific growth factors can be influenced by changes in PKC levels inside the cells (119). Evidence has accumulated to demonstrate that PKC plays a pivotal role in growth control and cellular differentiation, and that altered PKC activity has been implicated in malignant transformation (62, 73, 119-121).

Weinstein and his colleagues (119) have shown decreased levels of PKC (suggestive of PKC down-regulation) during human colon carcinogenesis and suggest that PKC plays a critical role in that process. Also, elevated PKC activity has been observed in breast tumor biopsies when compared to normal breast tissue (122). The enzyme has also been implicated in the dissemination of cancer cells. Thus, a strong correlation has been found between the basal level of membrane-bound PKC and metastatic ability of B16 melanoma sublines (123). Furthermore, treatment with PMA resulted in increased formation of metastases of SPI mouse mammary carcinoma cells and down-regulation of PKC inhibited any formation of metastases (123, 124).

PKC activators and inhibitors may also induce changes in cell proliferation and differentiation (120, 125-128). PMA-stimulated PKC activity was accompanied by increased cell proliferation in mouse histocytic sarcoma cells (120) and in murine leukemia cells (127). In other instances however, phorbol esters inhibited growth and induced differentiation of various human tumors (93, 94, 125-132). For example, PMA inhibited cell proliferation of the M5076 tumorigenic murine macrophage cell line (128), induced differentiation of human B chronic lymphocytic leukemia cells (130), and of normal human bronchial epithelial cells (132). In addition, PMA induced differentiation of human T-lymphoblastoid cell lines MOLT-4 (94) and MOLT-3 (133) along the T-cell lineage. PMA-induced differentiation of HL-60 cells along the monocytic pathway has also been reported (93).

It is important to note, however, that the apparent role of PKC in cell proliferation could be complicated by the possibility that the observed effects of phorbol esters might be

the result of down-regulation of the enzyme (134-138), rather than activation. Unlike DAG, the physiological activator, which is present only transiently in membranes and then degraded, phorbol esters are minimally degraded and persistently activate PKC (73, 139), and, thus, may prolong an ordinarily temporary cellular response. Such prolonged activation of PKC by phorbol ester would also elicit the degradation of the enzyme by proteases (6, 28, 29, 44). Under such circumstances, it may be argued that a particular effect is likely to be the result of PKC down-regulation. Instances of prolonged treatment with phorbol esters appeared to cause the induced differentiation of HL-60 cells (93), human promyelocytic leukemia cells (134), of K562 cells (135), of U937 cells (138), and inhibition of growth of Hep G2 cells (140). It may be inferred that alterations in cellular proliferation, or induction of differentiation might occur via PKC activation or down-regulation. It seems obvious from the above that the cell type may determine whether or not the end result of exposure of cells to PKC activators will be a stimulation of proliferation, inhibition of growth or induction of differentiation.

It is still not clear how PKC regulates cell proliferation and differentiation. The suggestion has been made, however, that modification in the PKC activity by phorbol esters may trigger a chain of events (62), including such processes as phosphorylation of various cellular components. These events may eventually influence the activities of specific transcription factors to affect gene expression. Activation of PKC by phorbol esters has been linked to the induction of several proto-oncogenes and other cellular genes, that have unique 5'-upstream DNA sequence segments that are responsive to activation by PKC (141, 142). Contained in the promoter regions of these genes are specific recognition/binding sites for enhancer proteins (for example, transcription factor AP-1) whose activities are stimulated by PMA through post-translational steps. AP-1, a family of protein complexes (mainly the products of c-*jun* and c-*fos)* (143-146), is believed to be a cellular enhancer-binding protein that can influence basal level transcription, and also can behave as an inducible factor targeted by the action of phorbol

ester-mediated changes in the cell (142). It has been observed that the protein complex, said to be *fos/jun* heterodimer, does bind to and stimulate transcription by its actions at the promoter regions of the DNA sequence which is responsive to phorbol ester (such sequences have been referred to as "TPA response elements" (TRE)) (146). Thus, phorbol ester-induced changes in the cell may regulate such transcription factors as AP-1 (142), which would subsequently cause transcriptional activation. For example, phorbol esters may trigger a chain of events including phosphorylation of targets inside the cell which may subsequently alter the activity of transcription factors (62, 140), such as AP-1. In fact, the observation has been made that partial down-regulation of PKC activity in NIH 3T3 cells abrogated transactivation of TRE by C-erbB-2 oncogenic protein (147). PKC-mediated phosphorylation of DNA methyltransferase (an enzyme which alters gene expression by changing DNA methylation patterns) (148), is another way by which the protein kinase may influence gene expression. It is clear that PKC might induce expression of certain genes to alter long-term cell proliferation.

Besides the effects of phorbol esters, use of PKC inhibitors, such as staurosporine (STA), H7, K252a or polymyxin B, has also implicated PKC in cell proliferation and cell cycle progression (149-159). H7-mediated inhibition of PKC blocked cell proliferation and induced differentiation of neuro 2a cells (149). STA reversibly blocked cultured, asynchronous, replicating cells of the human lung epithelial cell line, EFVX, in the G1 phase of the cell cycle, inhibiting replication (150). STA also induced cell cycle arrest and differentiation of K562 human leukemia cells (151). STA blocked cell cycle progression at G1 in nontransformed, human fibroblasts (152), and at higher concentrations, blocked both nontransformed and transformed FT210 mouse mammary carcinoma cells, HL-60, and CT10-2C-T1 cells at G2 (152, 153); UCN-01, a derivative of STA, also reportedly blocked human breast carcinoma cells from exit of G1 and entry into S phases of the cell cycle (154). Other PKC inhibitors have also reportedly blocked or inhibited cell cycle

progression. For example, the PKC inhibitors, K252a and RK-286C, blocked the cell cycle at G2 in rat diploid fibroblasts cells (155).

The mechanism by which PKC might stimulate or inhibit cell proliferation, therefore, may proceed via PKC-induced phosphorylation of its substrates. These targets may play an important role in cell cycle progression. It has been observed that cell regulatory molecules appear to be normally phosphorylated, albeit transiently, for brief periods during the cell cycle (160, 161).

1.3.3. RESEARCH OBJECTIVES

The role of PKC in the expression of nucleoside transport activity in human T-Lymphoblastoid CCRF-CEM cells

A substantial body of work suggests the involvement of PKC in cell proliferation, differentiation, and cell cycle progression. In contrast, only a few reports have suggested that PKC may be involved in the regulation of nucleoside transport activity in cells. For example, accompanying induction of differentiation of cells by phorbol esters are changes in nucleoside transporter activities (93- 95). Up to this time, very little is known about the mechanisms by which these changes in nucleoside transport are mediated by PKC during the process of differentiation. In one study, reduction in the number of high affinity NBMPR binding sites in coincidence with phorbol ester-induced PKC activation (96), while in another, prolonged exposure to phorbol esters induced Na^+-dependent nucleoside transport with a corresponding decrease in the facilitated diffusion of nucleosides in the same cells (93). In addition, as indicated earlier, results reported on the possible role of PKC in the regulation of nucleoside transporter are conflicting thus making further studies necessary to clear the obscurity that surrounds this subject.

Furthermore, insight into the role of PKC in regulation of NT activity may be important in the development of new approaches to improve the therapeutic effectiveness of nucleoside drugs. Nucleoside analogs, such as fludarabine, araC 2-chloro-2'-

deoxyadenosine, 2'-deoxycoformycin and 5-fluoro-2'-deoxyuridine (162-171) are employed in the treatment of cancers including leukemias. Transporter-mediated entry of these agents into tumor cells is an important determinant of their chemotherapeutic activity. Modulation of NT may, therefore, alter the overall therapeutic effectiveness of these drugs. Thus, it is important to understand how PKC activity may affect NT activity. For clinical purposes, it would be very useful if manipulation of PKC activity could result in enhancement of NT activity. For these reasons, the second part of this thesis was directed at investigating the role of PKC in the regulation the number of high affinity NBMPR binding sites and NT activity in CCRF-CEM cells (hereafter, CEM cells). To manipulate PKC activity in this project, PKC activators and inhibitors have been employed. The results of the first part of the thesis (see section 3.7.) showed that micromolar concentrations of STA produced non-PKC effects of this inhibitor that may overshadow inhibition of PKC. In view of this, concentrations of STA used in studies of NT activity were in the nanomolar range, to allow examination of the effects of PKC inhibition.

CEM cells are a T-lymphoblastoid cell line established from a patient with acute lymphoblastic leukemia (172). It has been reported that T-lymphocyte proliferation is coupled to PKC-dependent pathways (173), and that activation of PKC during ligand-receptor interactions may play a key role in the induction of certain T-cell functions in addition to proliferation (174).

For this project, CEM cells were suitable because they are easily maintained in culture, and also because they are leukemic cells, serving as a model for cells in which nucleoside analogs are used as therapeutic agents (162-171). NT in CEM cells is known to be mediated by an equilibrative, facilitated diffusion (*es*) process that is sensitive to NBMPR (102).

Phorbol esters and PKC inhibitors have been used as modulators of PKC activity to investigate PKC-mediated changes in high-affinity NBMPR binding site abundance as

well as changes in transporter-mediated adenosine influx. To further examine the involvement of protein phosphorylation in regulation of cellular abundance of NBMPR binding sites and NT activity, okadaic acid (OKA) and calyculin A (CLA), protein phosphatase inhibitors, have been employed in the course of this study. OKA is a polyether compound of a C_{38} fatty acid, produced by dinoflagellates and found in accumulated levels in marine black sponges from which it is isolated (175-179). Known receptors for OKA are protein phosphatases 1 and 2A (178-180). CLA is also obtained from sea sponges (177-179), but unlike OKA, which is several times more effective against phosphatase 2A than phosphatase 1, calyculin A is equally potent against both protein phosphatases 1 and 2A (177-179). The IC_{50} values for inhibition of phosphatase 1 are 1-2 nM for CLA, and 330-500 nM for OKA, and for phosphatase 2A, the values are both between 0.5 and 1 nM (179).

As part of the study, we have investigated the effects of PMA and STA on the proliferation of CEM cells, to determine whether PMA- and STA-induced changes in NT activity were related to cell growth. A tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (180-182), has been utilized in an assay to measure the proliferation of PMA- or STA- treated cells. In this assay, MTT is reduced to a colored reaction product, MTT formazan, a process that is catalyzed by mitochondrial dehydrogenases (180-182). Because the absorbance of the reduced dye can be shown to be proportional to cell number within a certain range, this assay can be used to determine the number of living cells in a culture (180-182).

Since prolonged incubation of cells with phorbol esters causes persistent activation, and consequently results in degradation and depletion of PKC (6, 28, 29, 44), studies were conducted as part of the thesis to assay for the levels of PKC in 16-hr PMA-treated cells. The studies determined whether PMA-induced PKC down-regulation occurred in parallel to the changes in NT activity observed in PMA-treated CEM cells.

2. MATERIALS AND METHODS

2.1. Materials

The following materials were purchased from the sources indicated: [^{3}H]myoinositol (80-120 Ci/mmol), ligand binding assay kit for Ins(1,4,5)P_3, PKC assay kit (Code RPN 77) and [^{32}P]ATP (3000 Ci/mmol) were from Amersham Canada Ltd. (Oakville, ON.); human thrombin, apyrase, neomycin sulphate, phorbol-12-myristate 13-acetate, prostacyclin, genistein, okadaic acid, calyculin A, 4α-phorbol-12,13-didecanoate, phorbol-12,13-dibutyrate, leupeptin, diagnostic protein assay kit and phenylmethylsulfonylfluoride were obtained from Sigma Chemical Co. (St. Louis, MO); K252A, and Fura 2-AM were from Calbiochem Corp. (San Diego CA); staurosporine was obtained from Sigma Chemical Co. (St. Louis, MO), and Boehringer Mannheim, (Montreal, PQ); HA1004 and H7 were from Sigma Chemical Co. (St. Louis, MO) and Calbiochem Corp. (San Diego, CA), bisindolylmaleimide was from Calbiochem-Novabiochem Corp.(La Jolla, CA), RPMI 1640 medium and fetal bovine serum were from GIBCO Ltd. (Burlington, ON). [2,8-^{3}H]Adenosine (30-39 Ci/mmol), [^{14}C(U)]sucrose (632.0 mCi/mmol or 360 mCi/mmol) and [^{3}H(G)]NBMPR (36 Ci/mmol) were purchased from Moravek Biochemical Inc. (Brea, CA), [^{3}H]$H_2$0 (5.95 Ci/mol) was obtained from ICN Biomedical Inc. (Irvine, CA). All other commonly used chemicals were of reagent grade.

Stock solutions of staurosporine, phorbol 12-myristate 13-acetate, phorbol 12,13-dibutyrate, 4α-phorbol 12,13-didecanoate, genistein, H7, were prepared in dimethyl sulfoxide (DMSO) and stored at -80°C or -20°C; calyculin A was dissolved in 50% (v/v) ethanol and stored at -20°C. When added to aqueous reaction mixtures, the final concentration of DMSO was 0.1% (v/v) (in CEM cells) or 0.4% (v/v) (in platelets), the final concentration of ethanol was 0.1% (v/v).

2.2. Preparation of platelet suspension

Blood was obtained from aspirin-free volunteers in 1/6 volume acid citrate dextrose (ACD). Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 300 x g at room temperature for 15 min. PRP was carefully transferred to a clear tube and centrifuged at 2500 x g at room temperature for 10 min to sediment platelets (183). Platelets were resuspended in Tyrode-HEPES buffer[1] containing apyrase (0.6 ADPase U/ml), and 0.35% (v/v) bovine serum albumin (BSA). For labeling platelets with [^{3}H]myoinositol, the buffer also contained 1 mM EGTA and 500 ng prostacyclin/ml (184).

2.3. Labeling platelets with [^{3}H]myoinositol

Human platelet suspensions (1.5-2.5 x 10^9/ml) were incubated with [^{3}H]myoinositol (100 μCi/ml) in the presence of 3 mM manganese chloride ($MnCl_2$) at 37°C for 4 h (185, 186). The platelet suspensions were subsequently washed with five volumes of the same Tyrode-HEPES buffer[1] containing apyrase (0.6 ADPase U/ml), BSA (0.35%, v/v) and 300 ng prostacyclin/ml and then centrifuged at 800 x g for 15 min (185, 186). The labeled platelet pellet was then resuspended in the same buffer containing apyrase (0.6 ADPase U/ml) and BSA (0.35%, v/v), but without EGTA or prostacyclin, at a platelet concentration of 8 x 10^8/ml.

2.4. Assay for [^{3}H]inositol phosphates

The [^{3}H]myoinositol-labeled platelet suspension was kept at 37°C for 45 min to allow equilibration of free [^{3}H]inositol and inositol incorporated into platelet

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[1]134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM glucose; pH 7.2

phosphoinositides (185). Inositol phosphate formation in platelets was assayed according to procedures of Lapetina and Siess (185). Briefly, portions (0.5 ml) of prelabeled platelets were incubated at 37°C and then stimulated with the appropriate agent for specified times varying from 5-120 s. In some experiments, the assay mixture contained 20 mM neomycin. Neomycin sulphate is reported to inhibit the phosphoinositidase, PLC, in intact platelets (91). For permeabilization, platelet suspensions were treated with 10 μg saponin/ml (187, 188). The assay mixture was similar to that used for ADP-ribosylation with pertussis toxin in saponin-permeabilized platelets (189-191) with slight modifications. It contained the following: NAD, 20 μM; ATP, 1 mM; $LiCl_2$, 10 mM; $MgCl_2$, 1.5 mM; dithiothreitol, 1 mM; thymidine, 10 mM. The concentrations of GTPγS, thrombin and STA were 10 mM, 1 U/ml and 2 μM, respectively, and the assay mixture was incubated at 37°C for 15 min.

Reactions were stopped with 1.88 ml of chloroform/methanol/HCl mixture (100/200/2, v/v/v), and phases separated by the addition of 0.6 ml each of chloroform and water, followed by centrifugation (800 x g, 15 min) (183, 185). The aqueous phases containing the inositol phosphates were neutralized by the addition of 1.0 M KCl (pH was monitored with litmus paper). Inositol phosphates were separated on Dowex 1-X8 anion exchange columns (formate form) (184, 185). Each sample loaded on the column was washed with water and then with buffer I[2], and subsequently, inositol mono-, bis-, and tris-phosphates were separated by eluting the columns with buffers II, III and IV in that order, respectively[2] (184, 185). Radioactivity was measured in the fractions using liquid scintillation counter.

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[2]Buffer I: 60 mM ammonium formate, 5 mM disodium tetraborate; Buffer II: 200 mM ammonium formate, 100 mM formic acid; Buffer III: 400 mM ammonium formate, 100 mM formic acid; Buffer IV: 800 mM ammonium formate, 100 mM formic acid.

2.5. Platelet ggregation

Aggregation studies were carried out on intact platelets according to published procedures (191). Samples (0.5 ml) of human platelet suspension, 3 $x10^8$/ml, in Tyrode-HEPES buffer[1], were placed in a two-channel aggregometer (Payton Associates Ltd.), with stirring and the temperature regulated at 37°C. Various agents, including STA, were added as the aggregation was followed.

2.6. Quantitation of Ins (1,4,5)P_3

The quantity of Ins(1,4,5)P_3 was determined by a competitive ligand binding procedure previously applied to neutrophil studies in the Wong laboratory (75, 88). Portions of human platelet suspensions (5-6 x 10^9/ml) in Tyrode-HEPES buffer[1] (184) were treated with various reagents for 15 s, and the reactions stopped by the addition of 0.2 ml of 40% trichloroacetic acid (TCA). The reaction mixture was centrifuged (12 000 x g, 5 min). The supernate was carefully removed, washed with 4 volumes of diethyl ether, neutralized by adding 1 M KOH (pH monitored with litmus paper), and the level of Ins(1,4,5)P_3 was assayed with a radioligand binding assay kit obtained from Amersham Canada Ltd. In brief, the principle of this assay is the competition between unlabeled Ins(1,4,5)P_3 and a fixed amount of [^{3}H]Ins(1,4,5)P_3 for binding sites on the Ins(1,4,5)P_3 binding protein from bovine adrenal glands. The major cross-reactivities reported by Amersham for Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 were 6.4% and 0.22% respectively.

2.7. Calcium fluorescence studies

Human platelet suspensions, 8 x 10^8/ml, in Tyrode-HEPES buffer[1], were incubated at 37°C with 1 μM Fura 2-AM for 45 min (192, 193). After incubation, the platelets were washed with the same buffer and centrifuged at 800 x g for 15 min, and

then resuspended in the same buffer at 3 x 10^8/ml. Washed platelets (2.0 ml) were exposed to various agents at 37°C, and were measured for changes in fluorescence at monochromator settings of 339 nm (excitation) and 505 nm (emission) (195). $[Ca^{2+}]_i$ was calculated as described previously (75, 88). In brief, $[Ca^{2+}]_i$ levels were calculated from the formula

$$[Ca^{2+}] = K_D (F-F_{min})/ (F_{max}-F)$$

where F is arbitrary fluorescent units and K_D (224 nM) is the dissociation constant for Ca^{2+} binding to fura-2 in an intracellular milieu. For cells suspended in buffer containing 1 mM Ca^{2+}, F_{max} was determined by lysing cells with 0.1 % (v/v) Triton X-100; F_{min} was determined by adding 20 mM Tris base and 2 to 4 mM EGTA to lysed cells (192), according to procedures established for use of quin2 (193, 194).

2.8. GTPase assay in human platelet membranes

Lysates of human platelets were obtained by rapid freezing and, prior to assay, thawing in 10 mM triethanolamine-HCl, pH 7.4 containing 5 mM EDTA. Immediately following the thawing, the lysed platelets were centrifuged for 20 min at 30 000 x g at 4°C. The pellets were washed twice in the same medium and finally suspended in 10 mM triethanolamine-HCl, pH 7.4, without EDTA.

High affinity GTPase activity was measured according to previously published procedures (195-197). Reaction mixtures contained the following: 0.1-0.3 μM [γ^{32}P]GTP (0.1 μCi/tube), 0.1 mM ATP, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM creatine phosphate, creatine kinase (0.4 mg/ml), and bovine serum albumin (2 mg/ml), in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 μl. Reactions were started

by adding platelet membranes (5-10 μg protein/tube) to the pre-equilibrated reaction mixture in the presence of thrombin (2 U/ml) or STA, at concentrations of 1.0 μM or 2 μM. The reaction incubation times ranged from 5 to 20 min at 25°C.

Reactions were stopped by adding 0.5 ml ice-cold sodium phosphate buffer (20 mM), pH 7.0, containing 5% (w/v) activated charcoal. The reaction tubes were centrifuged for 10 min at 10,000 x g at 4 °C, and 0.4 ml of the supernatant was transferred into scintillation vials for counting. High affinity GTPase activity was calculated from the difference between total ^{32}P formed in the presence of 0.3 μM GTP and that formed in the presence of 50 μM GTP. All assays were performed in triplicate.

2.9. Cell culture

Cultures of CCRF-CEM (CEM) cells used in this work were maintained at 37°C in unstirred suspension cultures in RPMI-1640 medium, pH 7.2, containing 10% (v/v) fetal bovine serum (FBS) (RPMI+FBS), in a humidified atmosphere of 5% (v/v) CO_2 in air. To ensure exponential proliferation, cell concentrations were kept at less than 6.0 x 10^5 cells/ml by dilution to 0.5 - 1.0 x 10^5 cells/ml with fresh medium every 2 - 3 days. After 30-35 subculture generations, new cultures were reinitiated from stocks cryopreserved in growth medium containing 10% (v/v) DMSO and 20% (v/v) FBS that were stored under liquid nitrogen.

2.10. Incubation of cells with modulators of PKC activity or inhibitors of protein phosphatases

Cell suspensions were diluted to 2.5 x 10^5 cells/ml in RPMI+FBS in the presence of the test agents, or vehicle. Except for okadaic acid (OKA) which was dissolved in 50% (v/v) ethanol, the rest of the test agents were prepared in 100% (v/v) DMSO. The final concentration of organic solvent in reaction mixtures was always 0.1% (v/v). The concentrations of the test agents used ranged from 1 nM to 40 μM; reaction mixtures

were incubated for intervals of 5 min to 24 h. After the desired incubation period, cells were pelleted at 90 x g for 5 min, and resuspended in transport medium (RPMIt)[3] at 4 x 10^7/ml and used immediately.

2.11. Proliferation of PMA- or STA-treated CEM cells

CEM cell suspensions (2.0 x 10^5 cells/ml) were incubated in RPMI+FBS in the presence or absence of 100 nM PMA or STA, for 16 h. Following this period, cells were washed and resuspended in fresh growth medium at 1 x 10^5/ml and incubated for a 4-day period. After each 24-hr interval, cells were enumerated electronically in a Coulter counter to determine cell proliferation, while remaining cells in the suspension were diluted to 2.5 x 10^5/ ml with fresh growth medium and incubated at 37°C, in a humidified atmosphere of 5% (v/v) CO_2 in air.

2.12. Measurement of adenosine influx

a) Measurement of time-courses of adenosine uptake

Adenosine (Ado) uptake time courses were determined at room temperature (22°C) in CEM cells suspended at 4 x 10^7/ml in RPMIt[3]. To initiate uptake, 100 µl of [^{3}H]Ado solution (1.0 µM) was rapidly added to an equal volume of cell suspension layered on 100 µl of paraffin oil/silicone oil in a microcentrifuge tubes. [^{3}H]Ado solutions were added sequentially to a group of tubes, according to metronome signals, and after 1,

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[3]RPMI-1640 containing 79.7 mM HEPES, and 56 mM NaCl, 290 ± 15 mOsmol; pH 7.4

3, and 5 s, uptake was terminated by centrifugation (15 000 x g, 30 s), which pelleted cells under the oil layer. Centrifugation under these conditions increased the interval of exposure of cells to permeant by 2 s (198).

To determine total pellet water volume and extracellular water space in cell pellets, cell suspension layered over oil was exposed to equal volume (100 µl) of [^{3}H]water or [^{14}C]sucrose was added for 3 s, followed by centrifugation (15 000 x g, 30 s). The difference between the former and the latter is a measure of the intracellular water volume (199). Also, labeled Ado present in the pellet extracellular water space was accounted for in each of the reported values for cell-associated ^{3}H permeant. Zero-time values were determined by adding 100 µl of [^{3}H]Ado solution containing 10 µM NBMPR to an equal volume of cell suspension layered over oil. Uptake data were analysed by linear regression to obtain progress curves of adenosine uptake into cells. The calculated slopes (during the first 7 s of uptake), which represented the initial rate of the uptake process, measured unidirectional, inward fluxes.

The [^{3}H]Ado content of cell pellets was measured as follows: contents of tubes above the oil layer were aspirated and the oil layer and walls of the tube were rinsed with water. Afterwards, oil layers were carefully removed by aspiration and 200 µl of 5% (v/v) Triton X-100 was added to cell pellets to lyse cells. Assay tubes with their contents were placed in counting vials and 4 ml of scintillation fluid (Ecolite) was added. Contents of vials were mixed and ^{3}H and ^{14}C activities were measured by liquid scintillation counting.

b) Measurement of kinetic constants (V_{max} and K_m) of ado influx

Time courses of [^{3}H]Ado uptake in CEM cells were measured as described under section (a) above for intervals of 3, 5 and 7 s. In these experiments, graded concentrations of [^{3}H]Ado (1-1000 µM) were used. For each concentration of Ado, inward fluxes were measured in the presence and absence of 5 µM NBMPR. Rates of Ado uptake into CEM cells that were treated or untreated for 16 h with 100 nM PMA or STA were determined.

2.13. Equilibrium binding of [^{3}H]NBMPR in CEM cells

Assay mixtures in 1.5 ml microcentrifuge tubes contained 2 x 10^6 cells layered over oil. In the assay mixture were graded concentrations (0.125 - 3 nM) of [^{3}H]NBMPR, with (for 'non-specific' binding) and without (for 'total' binding) 5 μM non-radioactive NBMPR. Following incubation at 22°C for 30 min, assay mixture was centrifuged (15 000 x g, 30 s) to terminate the reaction. The ^{3}H content of cell pellets, that is Bound, was determined as in the flux assay. The radioactivity associated with the supernate, that is Free, for each assay mixture was also measured. 'Non-specific' binding was determined from direct plot of Bound against Free of data obtained from binding studies in presence of unlabeled NBMPR and was used to calculate the actual 'non-specifically' bound ligand. Site-specific binding of NBMPR was therefore determined by subtracting 'non-specific' binding from 'total' binding. In some experiments, NBMPR equilibrium binding was measured in assay mixtures that contained a single high concentration [^{3}H]NBMPR (4.0 nM) with or without 5 μM unlabeled NBMPR.

2.14. Studies of the effects of STA and PMA on CEM cell proliferation

Cell suspensions (in RPMI+FBS) were centrifuged at 400 x g for 5 min and the pellets resuspended in R/.H/10F[4] at 8 x 10^4/ml. Portions (100 μl) of cell suspension were transferred to wells in multiwell plates and incubated at 37°C for 32 h, in a humidified atmosphere of 5% CO_2 in air. After this period, 50 μl of the clear medium above the cell

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[4]RPMI-1640 containing 2 mM HEPES and 10% (v/v) fetal bovine serum

layer was removed by aspiration from each of the wells and replaced with the same volume of medium containing graded concentrations of STA, 0 - 1000 nM (the control solution contained 0.1% (v/v) DMSO vehicle only), and then incubated for a further 16 h. Following this, cells were treated according to a modification of published procedures for tetrazolium-based assays for cellular viability (180-182). In brief, after the desired time, the plates were removed from the incubator and 30 µl of fresh MTT working solution (5 mg/ml in phosphate buffered saline solution[5]) added to each well. The plates were put in a container protecting wells from light and incubated for 2 h. Thereafter, the multiwell plates were then centrifuged, at 33 x g for 7 min. Most of the supernate from each well was aspirated and 50 µl of DMSO (spectral grade) was added to each well. The plates were then placed in the dark and agitated gently for 10 min and then a further 100 µl DMSO was added to each well with further gentle agitation of 2 min. Immediately following this, the absorbances were read on an ELISA reader at 540 nm.

In some studies, CEM cell suspensions in RPMI+FBS, at 2.5 x 10^5/ml, were treated with same concentration range (0-1000 nM) of PMA for 16 h. Afterwards, cells were washed and resuspended in R/H/10F[4] and transferred to wells in multiwell plates. They were incubated at 37°C for periods of 24, 48 72 and 96 h. Following these time intervals, 30 µl of freshly prepared MTT solution was added to each well containing PMA-treated cells, or control wells, and subsequently, cells were treated in the same way as before for color development and measurement of absorbances.

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[5]Phosphate buffered saline solution: 0.2 M NaCl, 8.06 mM $Na_2HPO_4 \cdot 7H_2O$, 1.47 mM KH_2PO_4, and 2.68 mM KCl

2.15. PKC assay

2.15.1. Processing of cells

CEM cells at 2.5 x 10^5/ml in RPMI containing 10% (v/v) FBS were treated with 100 nM PMA for 16 h. Subsequently, cytosolic and membrane fractions were prepared from cells according to procedure adapted from published ones (20-204). Briefly, cells were washed and resuspended in sonication buffer A[6]. Cells were then sonicated (56 W, 1 min, model W375) with short pulses. The disrupted cells were then centrifuged at 800 x g for 10 min, at 4°C, to remove unbroken cells and nuclei. The supernatant was centrifuged at 100 000 x g for 60 min, at 4°C, to separate cytosolic fraction from membrane fraction. The membrane pellet was solubilized in buffer B[7] for 60 min. Following the solubilization, the membranes were centrifuged at 4°C, at 100 000 x g for 60 min. The supernatant, referred to as the membrane or particulate fraction, was used for PKC assay, as was the cytosolic fraction from the cells.

2.15.2. Protein assay:

Protein concentrations of both cytosolic and membrane fractions were determined by the method of Bradford (206). The fractions were then diluted appropriately and used for PKC assay.

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[6]Buffer A: 20 mM Tris-HCl, pH 7.5 , 2 mM EDTA, 10 mM EGTA, 0.25 mM sucrose, 0.5 mM PMSF, 10 μg leupeptin/ml, 10 mM β-mercaptoethanol and 1 mM dithiothreitol.

[7]Buffer B: 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 0.25 mM sucrose, 0.5 mM phenylmethylsulphonyl fluoride, 10 μg leupeptin/ml, 10 nM β-mercaptoethanol and 1 nM dithiothreitol + 0.5% (v/v) Triton X-100.

2.15.3. Assay for PKC activity:

Activity of PKC was assayed with an assay kit from Amersham Canada Ltd., following the manufacturer's procedures. PKC activities associated with soluble and membrane fractions were measured as the incorporation of ^{32}P into a PKC-selective peptide. The assay mixture (75 µl) contained 50 mM TrisHCl, pH 7.5, 12 mM calcium acetate, 8 mole % lα-phosphatidyl-l-serine, 24 µg phorbol 12-myristate 13-acetate/ml, 900 µM peptide, 30 mM dithiothreitol, 45 mM Mg acetate, 150 µM ATP and 10 µCi/ml [^{32}P]ATP (500 cpm/pmol) and the enzyme (cell) fraction (15-25 µg protein/ml). The reaction was started by the addition of 25 µl of combined 150 µM MgATP and [^{32}P]ATP (500 cpm/pmol) to 50 µl of protein solution.

Following incubation for 15 min, at 25^{o}C, the reaction was stopped by the addition of 100 µl of stopping reagent (a dilute, acidic, reaction-quenching reagent supplied with the kit) to each assay tube. Washing of the radioactivity-containing papers was done as follows. First, binding papers (2.5 cm by 2.5 cm, supplied by the manufacturer) were placed on glass filter surfaces which were connected to a vacuum source. 125 µl of terminated reaction mixture was pipetted onto peptide binding paper while the vacuum source was off. After the radioactive mixture had soaked into the paper, the vacuum was turned on and the washing started. 60 ml of 5% (v/v) acetic acid was used for washing each paper. Following washing, the papers were allowed to dry prior to transferring them into scintillation vials. Radioactivity was counted after 4 ml counting fluid (Ecolite) was added to each vial.

Specific PKC activities for soluble and membrane fractions were each expressed as pmol phosphate incorporated into peptide per min per mg protein.

3. RESULTS

3.1. NON-PKC EFFECTS OF STA ON INOSITOL PHOSPHATE SIGNAL TRANSDUCTION IN HUMAN PLATELETS

3.1.1. Effect of STA on $[Ca^{2+}]_i$ of human platelets[8]

STA concentrations up to 4 μM induced a weak transient elevation of $[Ca^{2+}]_i$ in platelets suspended in Ca^{2+}-free medium (Fig. 1A, traces a, b) which was not observed in vehicle-treated cells (trace c). Subsequent addition of Ca^{2+} did not affect the level of $[Ca^{2+}]_i$. The apparent increase in signal was attributed to extracellular Ca^{2+} binding to small amounts of extracellular fura-2 since this signal was quickly depressed by the addition of Tris-EGTA (traces b, c). Thrombin on the other hand elevated $[Ca^{2+}]_i$ in platelets from nearly 0.1 μM to > 0.5 μM (trace a). The rise in thrombin-induced $[Ca^{2+}]_i$ peaked at less than 10-15 s and declined gradually to resting levels in several minutes. In the presence of 1 mM Ca^{2+}, STA induced an equally transient elevation in $[Ca^{2+}]_i$ (trace d). In this case, pretreatment with STA augmented the effect of thrombin on platelet $[Ca^{2+}]_i$. The extent and duration of the thrombin-mediated rise in $[Ca^{2+}]_i$ was increased compared with the response induced in cells treated with thrombin alone (trace e).

Entry into platelets of Mn^{2+} and subsequent quenching of fura-2 fluorescence was employed as an indirect measurement of Ca^{2+} influx (Fig. 1B) (75, 88). Added to resting platelets, Mn^{2+} produced a basal rate of quenching due to entry via leak channels. In agreement with other published results, activation of cells by thrombin accelerated Mn^{2+} entry (208).

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[8]Results of this study have been published (ref. 207).

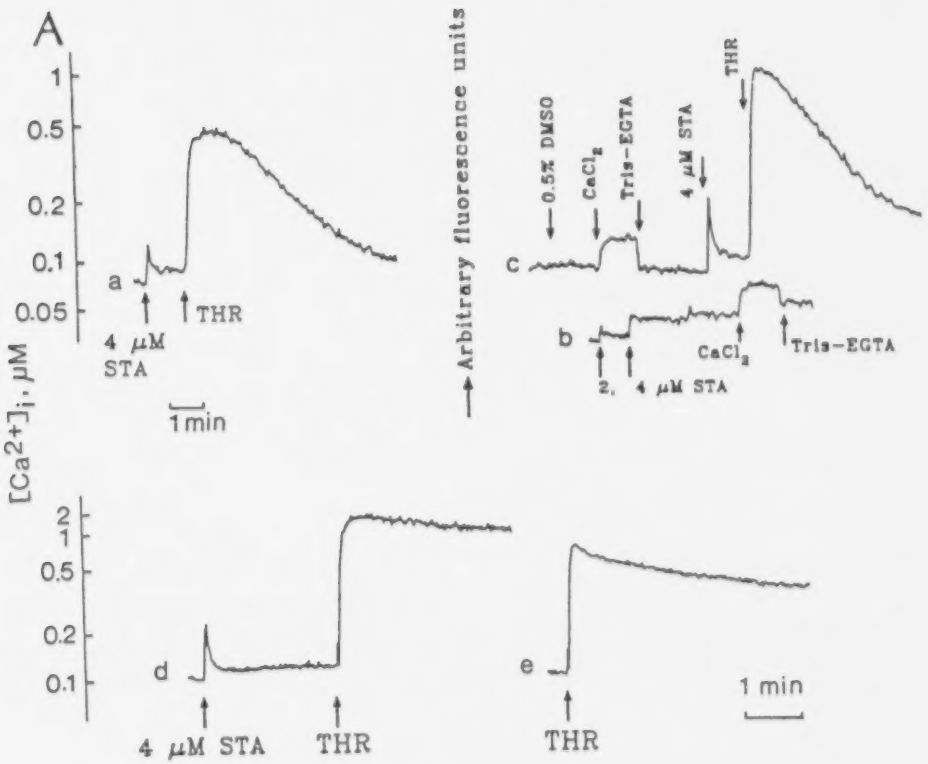

FIG. 1 (A). The effect of STA on $[Ca^{2+}]_i$ of human platelets. Human platelets, preloaded with fura-2 AM and suspended in Tyrode-HEPES buffer[1], were treated with STA and other agents in the order shown in the figure. Final activity and concentrations were 1 U/ml thrombin (THR), 1 mM $CaCl_2$, 25 mM Tris-base and 2 mM EGTA. Fluorescent measurements and calculation of $[Ca^{2+}]_i$ (except for traces b and c) were carried out as outlined in "Materials and Methods". In traces (d) and (e), the medium was supplemented with 1 mM $CaCl_2$. Results shown are representative of 4 experiments.

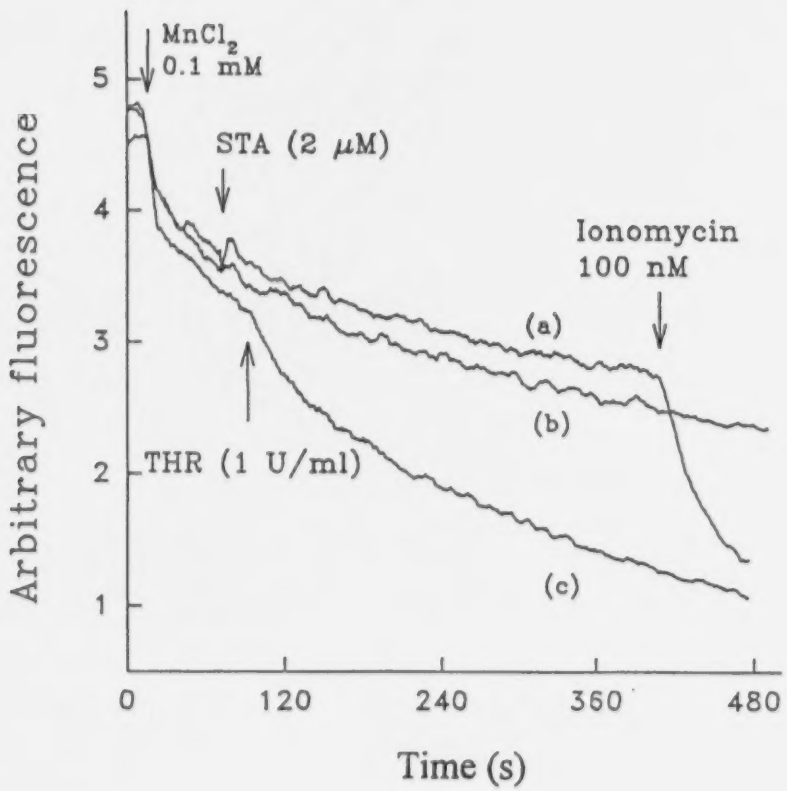

FIG. 1 (B). Kinetics of Mn^{2+} quenching of fluorescence in thrombin- and STA-treated human platelets. Fura-2 loaded platelets were stimulated with reagents in the following order: trace (a), Mn^{2+}, STA, ionomycin; trace (b), Mn^{2+}; trace (c), Mn^{2+}, thrombin. The excitation wavelength used was 360 nm. Results shown are representative of 3 experiments.

STA, at 2 μM, did not affect the basal rate or Mn^{2+} entry mediated by ionomycin. Together the results of Fig 1A and B suggest that STA induced a limited release of $[Ca^{2+}]_i$ without any accompanying influx of extracellular Ca^{2+}.

3.1.2. Time-course of $[^3H]InsP_3$ formation induced by STA or thrombin in intact human platelets

Activity of PLC in intact platelets prelabeled with $[^3H]$myoinositol was assayed as an elevation of $[^3H]InsP_3$. Isomers of $InsP_3$ were not resolved by the anion exchange chromatographic procedure that was employed. Thrombin, a physiological agonist, induced rapid hydrolysis of PIP_2; $InsP_3$ levels were maximally elevated at the earliest time sampled, 10 s following addition of thrombin to platelets. Subsequently, $InsP_3$ levels declined to basal levels (Fig. 2). Concentrations of 1-2 μM STA also induced rapid formation of $InsP_3$ which plateaued at 15-20 s but remained elevated after 2 min. The maximum levels of $InsP_3$ observed were similar to that found for thrombin. The STA response was dose-dependent with significant $InsP_3$ production occurring at 0.5 μM or higher concentrations of STA (Fig. 3). When platelets were treated with 20 mM neomycin, an inhibitor of PLC (91), STA-mediated $InsP_3$ formation was abolished (Fig 3). This result suggests that STA caused increased $InsP_3$ levels via activation of the PLC-PIP_2 hydrolytic pathway.

3.1.3. Formation of $[^3H]InsP_3$ in permeabilized human platelets treated with STA or other agents

PLC activity was assayed in saponin-permeabilized, $[^3H]$inositol-labeled platelets by measurement of the levels of $[^3H]InsP_3$ formed. Thrombin, STA (2 μM), and GTPγS, a nonhydrolyzable analog of GTP, all caused formation of significant levels of $InsP_3$ (Fig. 4). The presence of GTPγS and STA together caused production of relatively higher levels

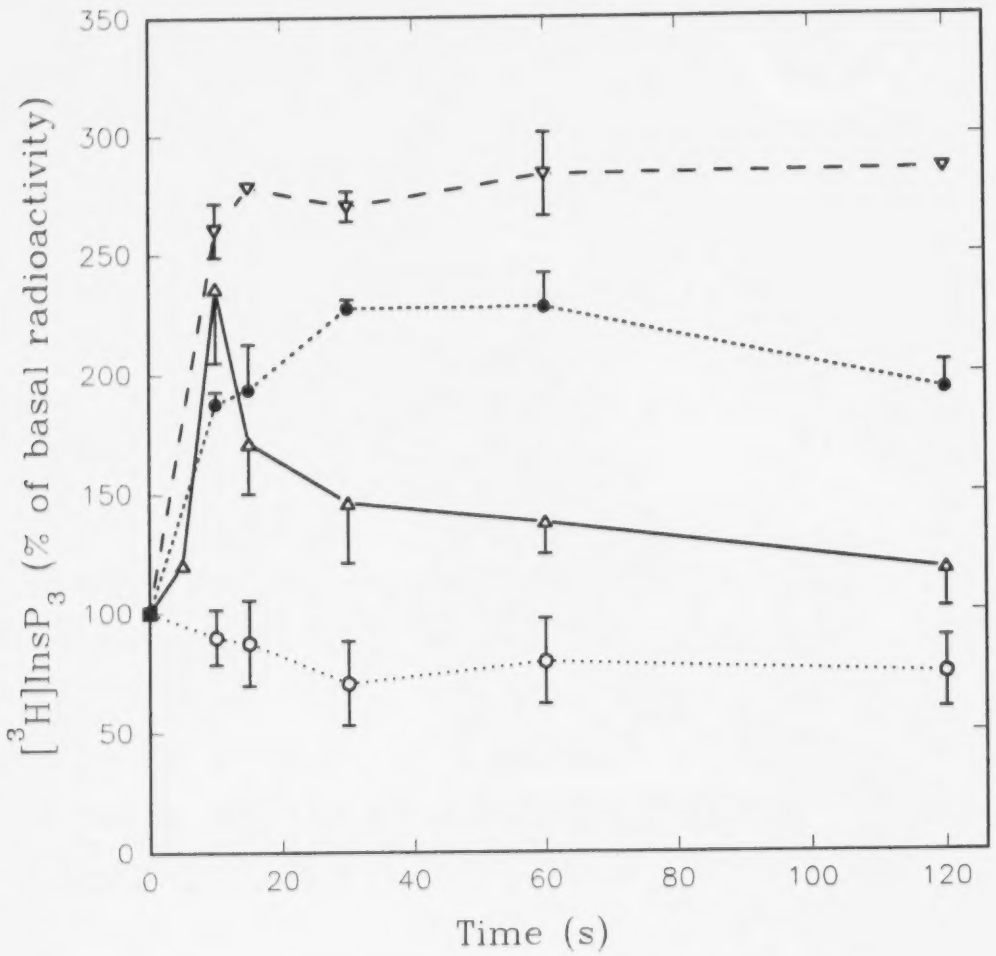

FIG. 2. Time-course of $[^3H]InsP_3$ formation in human platelets treated with thrombin or STA. Human platelets preloaded with [^{3}H]myoinositol were incubated with thrombin, 2 U/ml (Δ), STA, 1 μM (●), 2 μM (∇) or 0.4% (v/v) DMSO (vehicle) (O), for up to 120 s. Reactions were stopped and [^{3}H]inositol phosphate levels determined as described in "Materials and Methods". Results were calculated as % of basal (control) radioactivity in vehicle-treated platelets at time zero. Each point represents the mean ± S.E.M. for 4 separate experiments, each performed in triplicate.

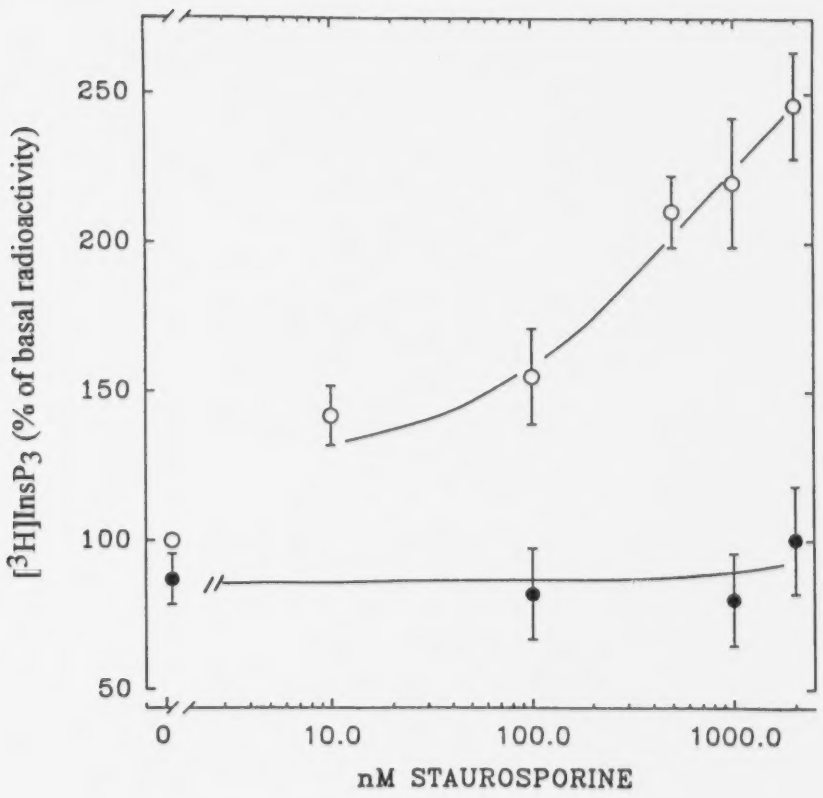

FIG. 3. Concentration-dependence of STA on human platelet [^{3}H]InsP$_3$ levels and the effect of neomycin. [^{3}H]InsP$_3$ recovered from platelets after 60 s incubation with STA (O) or STA plus 20 mM neomycin (●) were calculated as % of basal radioactivity in vehicle (0.4% (V/V) DMSO)-treated platelets. Each point is the mean ± S.E.M. of at least 3 separate experiments, each performed in triplicate.

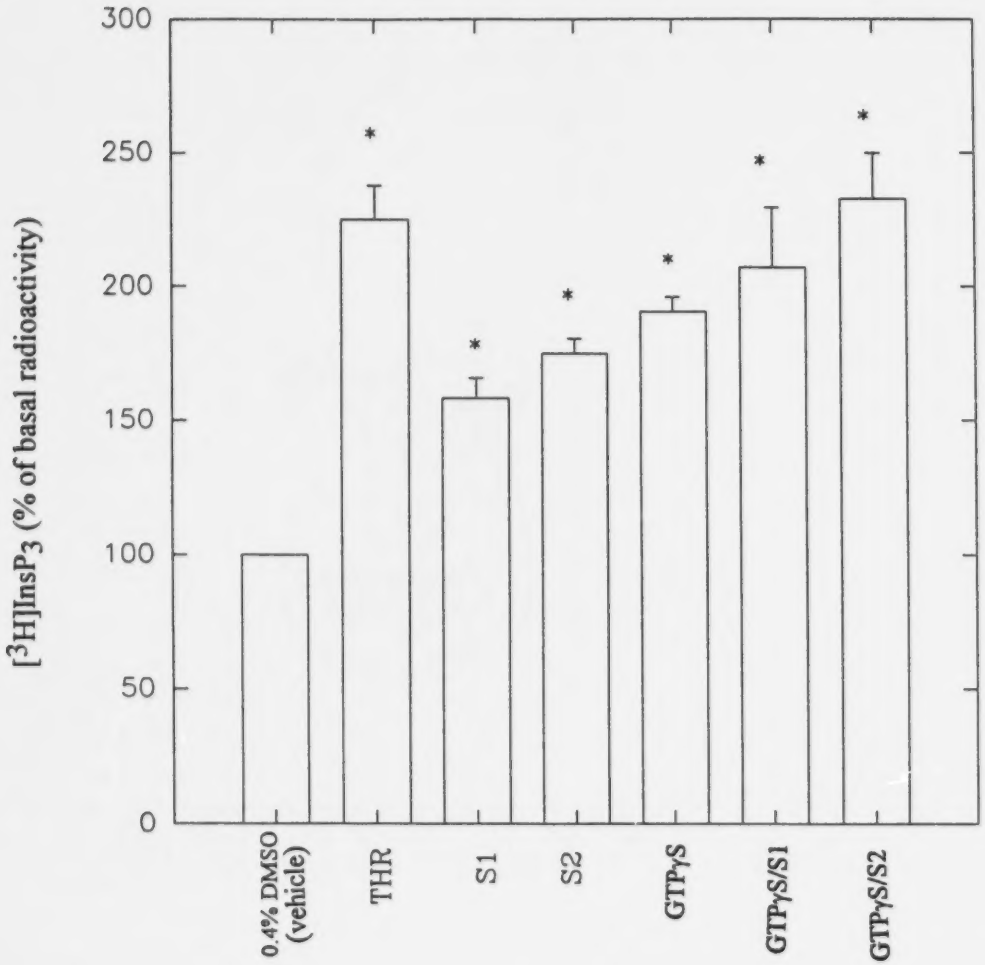

FIG. 4. Formation of $[^3H]InP_3$ in saponin-permeabilized human platelets treated with STA. Human platelets preloaded with [^{3}H]myoinositol, were treated with saponin followed by thrombin, 2 U/ml, 0.4% (V/V) DMSO (vehicle), STA, 1 μM (S1), 2 μM (S2), GTPγS, 10 mM or STA plus GTPγS, for 15 min. Reactions were stopped and [3H]inositol phosphate levels determined as described in "Materials and Methods". Each result represents the mean ± S.E.M. of 4 separate experiments, each performed in triplicate. *Significantly different from control (vehicle-treated) at $P<0.05$.

of $InsP_3$ than when either one was used alone. The combined effect was less than additive and did not suggest a synergism.

3.1.4. Formation of Ins(1,4,5)P_3 in thrombin- and STA-treated human platelets

The hydrolysis of PIP_2 produces Ins(1,4,5)P_3 and 1,2-diacylglycerol (4). Ins(1,4,5)P_3 was specifically quantified with a competitive binding assay (Amersham kit). Results show that 2 μM STA elevated Ins(1,4,5)P_3 levels to about 50-60% of levels induced by thrombin (Fig. 5). The fact that lower amounts of Ins(1,4,5)P_3 were measured by this assay than found by anion exchange chromatography could be due to the presence of other isomers of Ins(1,4,5,)P_3 present in the $InsP_3$ fraction eluted from columns.

3.1.5. GTPase activity of human platelet membranes treated with STA or other agents

Since the above results suggested that STA might be directly affecting a molecular target along the PLC-PIP_2 pathway, and possibly a G protein, further studies were carried out to assay for G protein via GTPase activity in the presence of STA. The results showed that STA caused a linear increase with time in the rate of GTP hydrolysis in crude platelet membranes. The increase in GTPase activity induced by STA was concentration-dependent (Fig. 6). In contrast, other PKC inhibitors, such as K252a, and H7, did not induce any significant activity of GTPase in platelet membranes at the concentrations tested.

3.1.6. Effect of STA on human platelet aggregation

Activation of platelets by agonists can be divided into two phases. The initial phase, called the primary aggregation, is marked by a change in cell shape (reflected by an

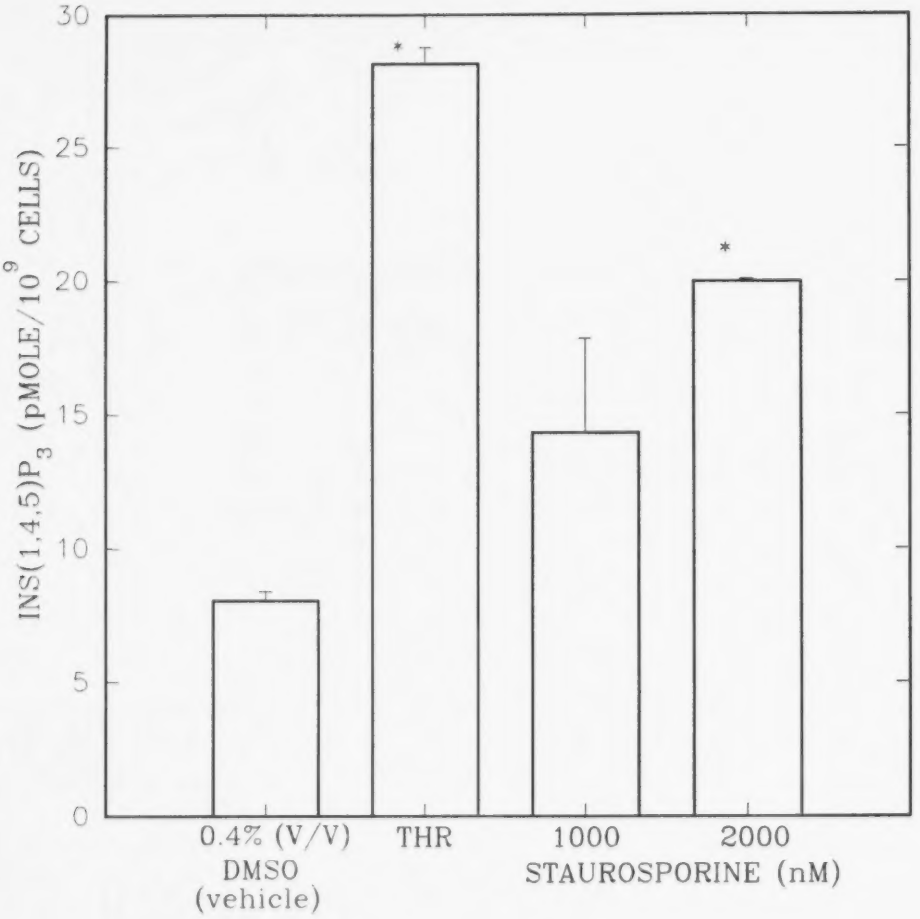

FIG. 5. Ins(1,4,5)P_3 formation in STA- or thrombin-treated human platelets. Ins(1,4,5)P_3 levels in platelets incubated for 15 s with thrombin, STA or 0.4% (v/v) DMSO (vehicle), were quantified by the Ins(1,4,5)P_3 ligand binding assay as described in "Materials and Methods". Results represent the mean ± S.E.M. of 3 separate experiments, each performed in duplicate. *Significantly different compared with control (vehicle-treated) values ($P<0.05$) by the Student's paired *t*-test.

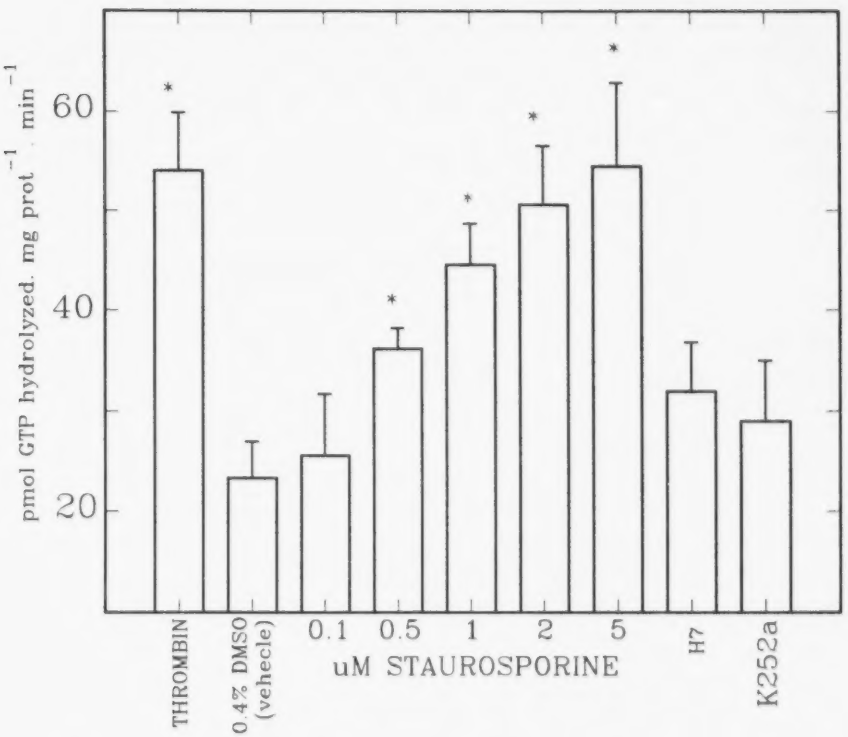

FIG. 6. GTPase activity of human platelet membranes treated with STA or other agents. Platelet membranes were prepared and incubated at room temperature for 10 min in the presence of thrombin (2 U/ml), 0.4% (V/V) DMSO (vehicle), STA (0.1 to 5 μM), K252A (2 μM) or H7 (20 μM) and GTPase activity measured as described in "Materials and Methods". Results represent the mean ± S.E.M. of 4 separate experiments, each performed in triplicate. *Significantly different from control (vehicle-treated) value (P<0.05).

increase in light scattering), and reversible aggregation of cells (measured as a decrease in light scattering) (Fig. 7). A secondary phase following the initial primary aggregation, is characterized by irreversible aggregation (Fig. 7) and secretion (209). Thrombin stimulation of platelets triggered a small shape change followed by rapid aggregation (Fig. 7, trace a). STA by itself did not induce any significant aggregation (traces b and c). DMSO vehicle also had no effect (trace d). When platelets were preincubated with STA (1 μM and 2 μM) for several min before being stimulated with thrombin, the extent of the ensuing shape change was enhanced (traces b and c). Furthermore, 2 μM STA but not 1 μ M decreased the rate of aggregation caused by thrombin (trace c).

Compared with thrombin, PMA (40 nM) induced platelets to undergo a slower but similar sequence of shape change and aggregation (trace e). If thrombin was added to cells about 2 min after PMA (prior to onset of PMA effect), the thrombin effect on platelet responses dominated (trace f). As anticipated, 2 μM STA suppressed responses mediated by PMA (trace g); the consecutive addition of STA, PMA and thrombin completely inhibited thrombin-induced platelet aggregation but not shape change (trace g).

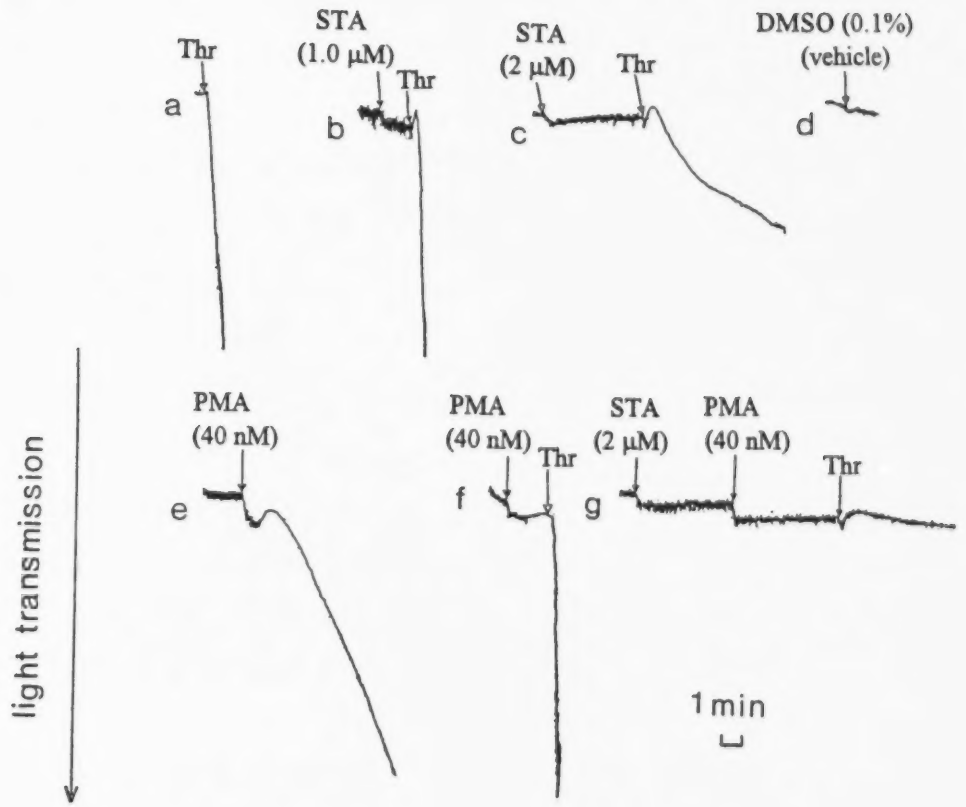

FIG. 7. Effect of STA alone or in combination with other agents on human platelet aggregation. Platelet aggregation was measured as outlined in "Materials and Methods". Reagents were added directly to 0.5 ml platelets to final concentrations indicated in traces. Thrombin activity was 1 U/ml. DMSO (vehicle) was present at a final concentration of 0.4% (v/v). Traces are representative of 3 separate experiments.

3.2. MODULATION OF PKC ACTIVITY AND ITS EFFECTS ON PROLIFERATION OF CEM CELLS

3.2.1. Effects of PMA and STA on proliferation of CEM cells

a) Assessed as cell counts by Coulter counter

CEM cells (2.5 x 10^5/ml) in RPMI 1640 containing 10% (v/v) FBS (RPMI + FBS), were treated with 100 nM PMA or STA , or 0.1% (v/v) DMSO vehicle, for 16 h. Cells were subsequently washed and resuspended in drug-free growth medium and subcultured during a 4-day period. Cell counts on day 0 (16 h after drug-treatment) and on each subsequent day are represented in Fig. 8A. The doubling time for vehicle-treated cells was 21.5 h. The doubling time of untreated cells was in the range of 18 to 25 h (data not shown). Thus treatment with the vehicle does not affect the rate of doubling of cells. The 16-h treatment of CEM cells with PMA did not alter cell proliferation rate. This is evident by the similar slopes of the curves of cell numbers against time for vehicle- and PMA-treated cells between -16 h (beginning of treatment) and 0 h (Fig. 8A). The slope of the curve for PMA-treated cells from 0 to 96 h is, however, substantially lower than that for control cells indicating that PMA-treated cells doubled at a far slower rate than control cells. The doubling time for the PMA-treated cells from 0 to 96 h was more than 132 h. This means that during 16-h treatment with PMA, CEM cells proliferated at the same rate as control cells. However, following the 16-h treatment period, PMA-treated cells proliferated at a much slower rate than control cells (about 6 times slower).

The pattern of proliferation of STA-treated cells was much different. The cell number verses time curve for STA-treated cells shows a negative slope from -16 to 48 h (Fig. 8A) which indicates a net loss of cells during and after treatment. The negative slope is an indication that cell death predominated over cell proliferation during the 16 h

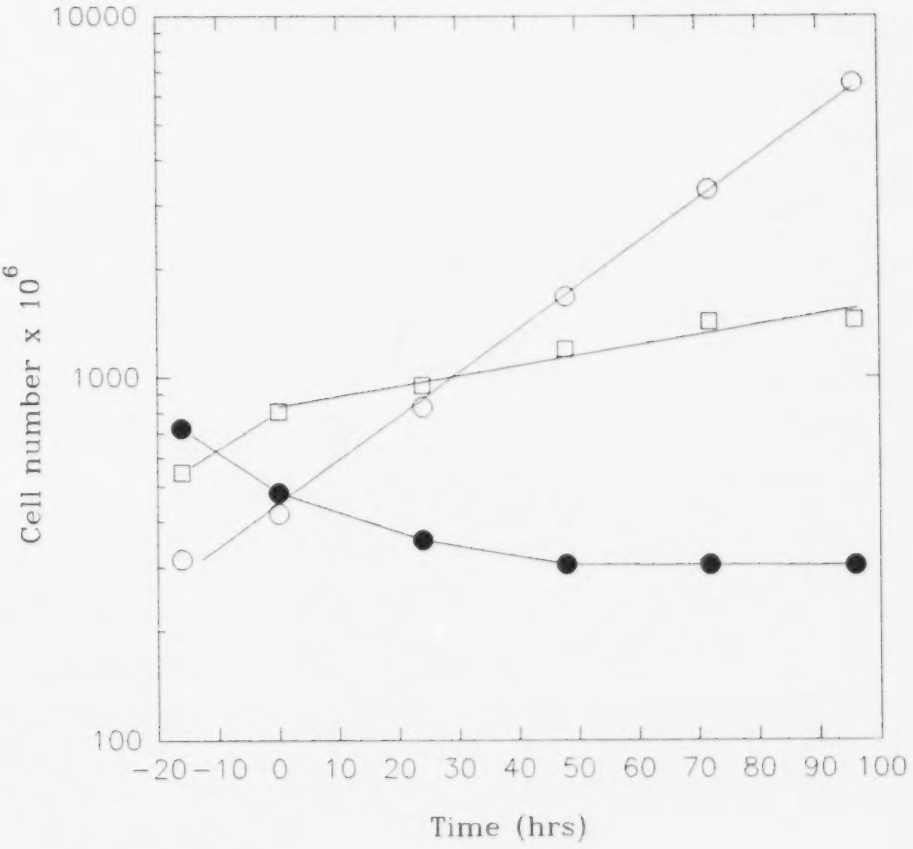

FIG. 8 (A). Effect of treatment with PMA or STA on proliferation of CEM cells assessed by Coulter counter. CEM cell suspensions (2.5 x 10^5/ml. were treated with 0.1% (v/v) DMSO (vehicle) (O), or 100 nM each of PMA (□) or STA (●) for 16 h. Subsequently, portions of cell suspensions were removed for enumeration of cells in a Coulter counter and the remaining cells washed and resuspended in drug-free medium at 1 x 10^5/ml. Cumulative cell numbers are plotted against time of incubation. Each point is the mean of 12 replicate measurements in 2 separate experiments.

treatment and a short while afterward, that is STA was cytotoxic. The rest of the curve for the STA-treated cells shows no change in slope suggesting that neither cell proliferation nor cell death predominated during this period (50 - 96 h).

b) Assessed by MTT assay

The results of Fig. 8A showed that PMA or STA caused a decline in the rate of cell proliferation or caused cell death. To understand further the PMA- or STA-mediated changes in cell proliferation rate, tests of the viability of PMA- or STA-treated cells were carried out. The test of cell viability was based on the ability of live cells to convert a tetrazolium salt, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), to a colored MTT formazan through the activity of mitochondrial dehydogenases (180-182). Previous studies in this laboratory have shown that the development of color from MTT formazan is a linear function of cell number, within the range of cell concentrations present in these experiments (Gati, W. P., unpublished results).

During 16-h treatment of CEM cells with PMA, there was no change in cell viability, according to the MTT assay, indicated by the horizontal line shown in Fig. 8B. This result agrees with the observation made on the effect of PMA on cell proliferation during 16-h treatment period (-16 to 0 h) measured with a Coulter counter (Fig. 8A). However, following 16-h treatment with PMA, cells showed a consistent decline in viability (Fig. 8B). The decline was dependent on the concentration of PMA, and the length of time cells were cultured subsequent to the 16-h treatment with PMA. Cells treated with high concentrations of PMA, (100 nM and over) tended to show a higher decline in proliferation rates. After 72 or 96 h, the number of viable cells was less than 30% of control. The results of the effect of STA on viable cell numbers in the MTT assay, however, showed that during 16-h incubation of cells with STA concentrations higher than 10 nM, there was a dose-dependent decline in viable cells to nearly zero

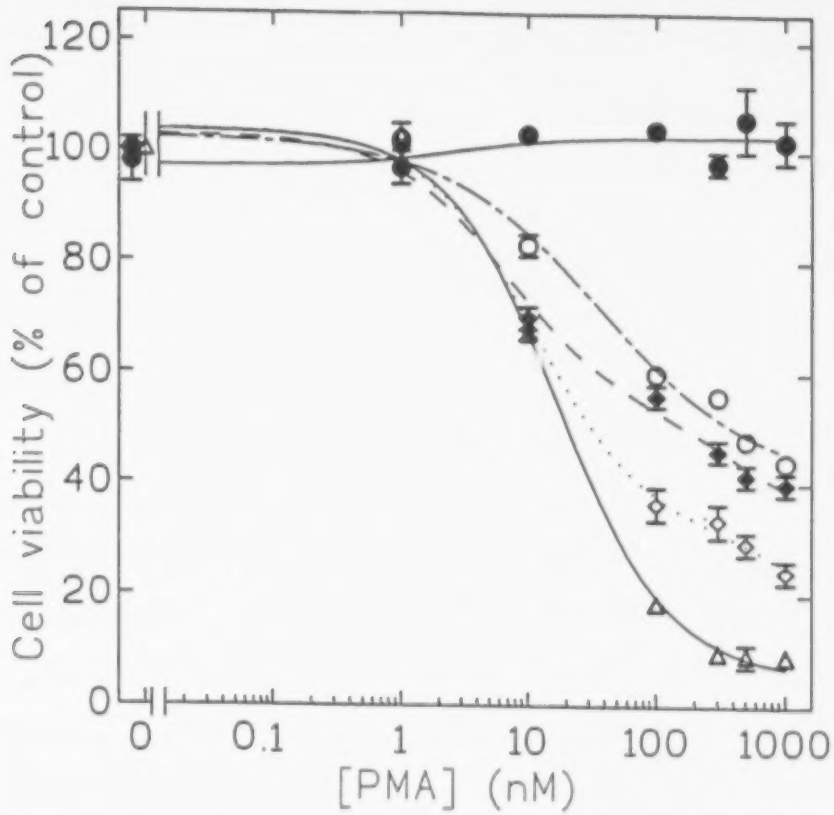

FIG. 8 (B). Effect of PMA on viability of CEM cells assessed by MTT assay. Samples (100 µl, 8 x 10^4/ml) of cell suspension in R/H/10F solution (RPMI-1640 + HEPES + 10% FBS, pH 7.4), were transferred to wells in multiwell plates and incubated at 37°C for 32 h. After this period, 50 µl volumes of the clear medium above the cells in each well were removed by aspiration. The same volumes of PMA, 0 - 1000 nM (0 nM solution contained 0.1% (v/v) DMSO vehicle only), were added to the designated wells; cells were then incubated for a further 16 h (●). Subsequently, cells were treated with freshly prepared MTT reagent for color development as described in "Materials and Methods". In other experiments, cells were pretreated with the same concentrations of PMA (0 - 1000 nM) for 16 h. PMA-treated cells were then transferred to wells in multiwell plates and incubated for 24 (O), 48 (◆), 72 (◇) or 96 h (Δ). Thereafter, the MTT assay was performed also as described in "Materials and Methods". Each point is the mean ± S.E.M. of 12 replicate measurements in 2 separate experiments.

percent of control (Fig. 8C). This result corroborates the findings obtained for the effect of the PKC inhibitor on cell proliferation, measured by Coulter counting, the results of which showed that there was a net cell loss during 16-hr treatment of CEM cells with STA (-16 h to 0 h of curve) (Fig. 8A).

3.2.2. PKC ASSAY IN PMA-TREATED CEM CELLS

Experiments were conducted to directly measure PKC activity in CEM cells previously treated with 100 nM PMA for 16 h. PKC activity was assayed in cytosolic and membrane fractions of cells using a PKC assay kit purchased from Amersham Corp. The PKC present in cell preparations catalyze the transfer of the γ-phosphate of adenosine-5'-triphosphate to the threonine residue on a PKC-specific peptide substrate. Results from this assay showed that after 16-h treatment of cells with 100 nM PMA, PKC activities in both cytosolic and membrane fractions were significantly reduced to 4% and 2% of PKC activity in control (16-h, 0.1% DMSO, vehicle-treated) cells (Fig. 9). Specific PKC activities in control cells were about 97.2 and 73.4 pmol p/min/mg protein in cytosolic and membrane fractions, respectively (Fig 9), while specific PKC activities in cells treated for 16 h with 100 nM PMA were about 3.9 and 1.5 pmol/min/mg protein for cytosolic and membrane fractions, respectively. This result is in keeping with the current understanding that prolonged exposure of cells to phorbol esters results in down-regulation of PKC (6, 28, 29, 44).

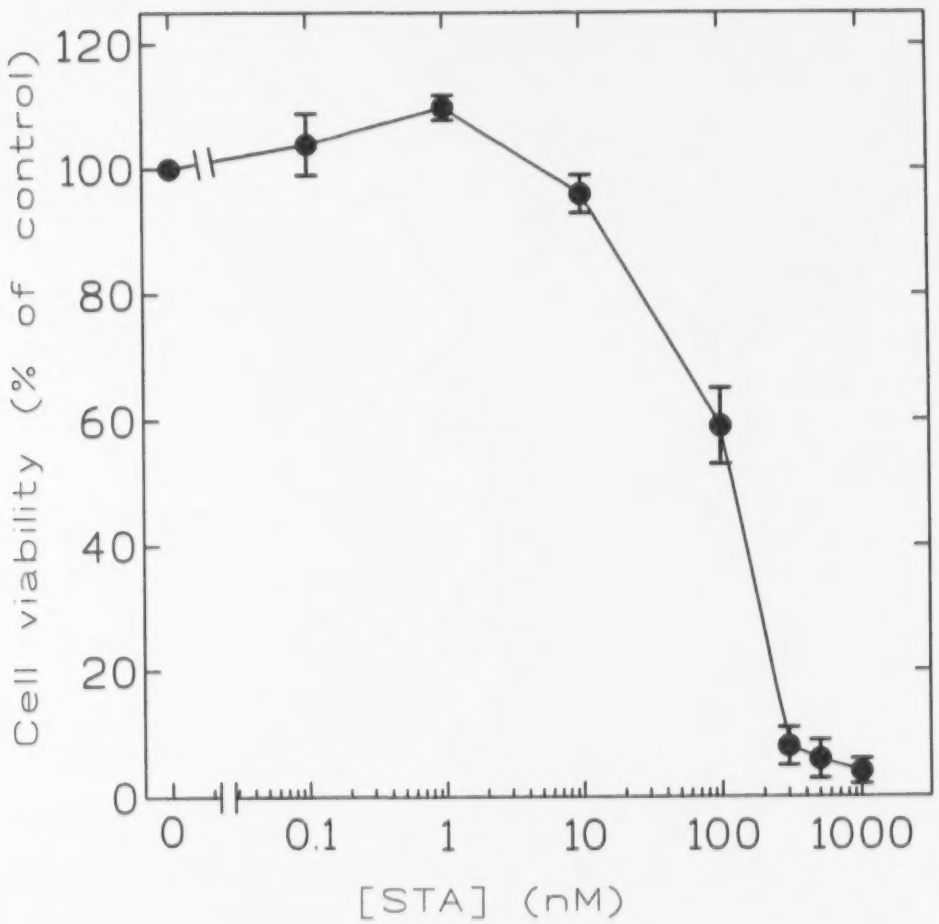

FIG. 8 (C). Effect of STA on viability of CEM cells assessed by MTT assay. Portions of cell suspension (100 µl, 8 x 10^4/ml) in R/H/10F, were transferred to wells in multiwell plates and incubated at 37°C for 32 h. Subsequently, 50 µl volumes of the clear medium above cells in each well were removed by aspiration. The same volume of STA, 0-1000 nM (0 nM solution contained 0.1% (v/v) DMSO, vehicle only), were added to the designated wells. Cells were then incubated for a further 16 h. Following this, cells were then treated with freshly prepared MTT reagent for color development as described in "Materials and Methods". Each point is the mean ± S.E.M. of 12 replicate measurements in 2 separate experiments.

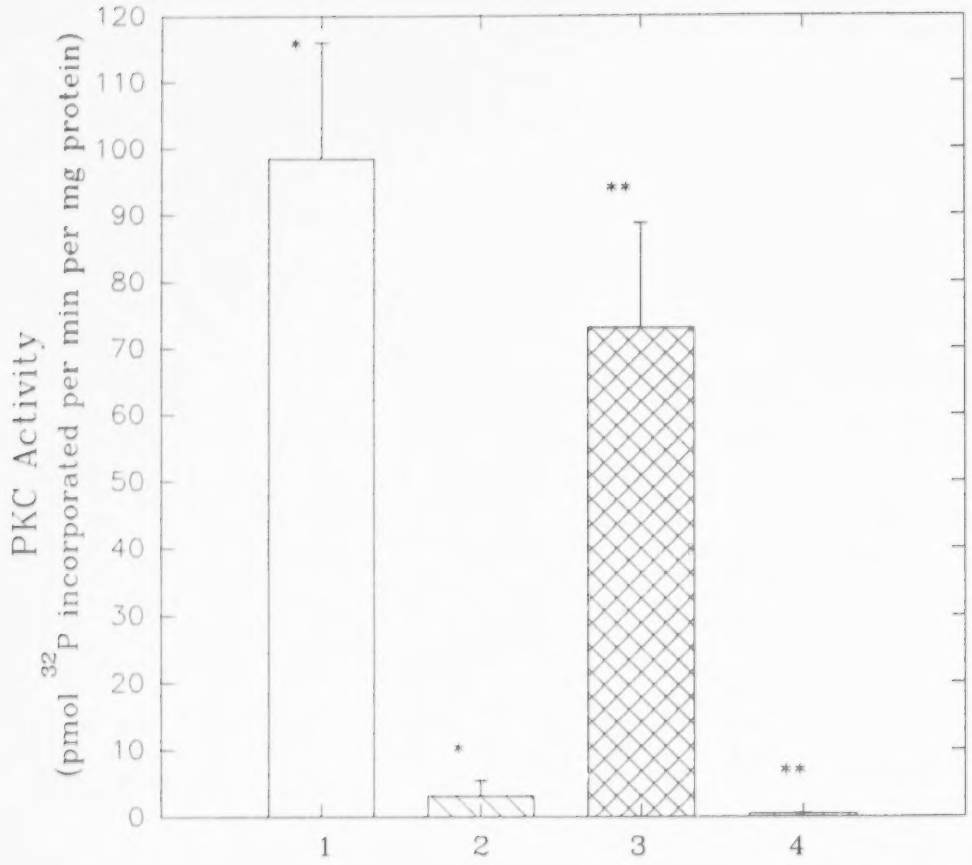

FIG. 9. PMA-induced down-regulation of PKC in CEM cells. CEM cells were treated with 0.1% (v/v) DMSO (vehicle) (bars 1 and 3) or 100 nM PMA (bars 2 and 4), for 16 h. Subsequently, cells were washed and resuspended in sonication buffer A. Cytosolic (bars 1 and 2) and membrane (bars 3 and 4) fractions were prepared and PKC activity was assayed in each fraction as described in "Materials and Methods". Results are the means ± S.E.M. of 4 experiments, each performed in triplicate. (*) or (**) indicates that the difference between the PKC activities of pairs of cytosolic or membrane fractions, respectively, were significant, ($P<0.02$ or $P<0.05$).

3.3. EFFECT OF PKC MODULATORS ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND INWARD FLUXES OF ADENOSINE IN CEM CELLS

Changes in the number of NBMPR binding sites and transporter activity which occurred following treatments of CEM cells with phorbol esters or PKC inhibitors were determined. Cells were previously treated with the PKC modulators prior to measurements of equilibrium binding of [^{3}H]NBMPR or [^{3}H]Ado influx.

3.3.1. Effect of treatment of cells with PMA on equilibrium binding of NBMPR in CEM cells

CEM cells were cultured in the presence of 0.1% (v/v) DMSO (vehicle) for 16 h, or 100 nM PMA for 0.5 - 24 h prior to measurements of equilibrium binding of NBMPR. Values of cellular abundance (B_{max}) of NBMPR binding and the equilibrium dissociation constant, K_D, in control (vehicle- treated) and in untreated cells were 17.4 x 10^4 molecules/cell, 0.39 nM, and 17.3 x 10^4 molecules/cell, 0.38 nM respectively (result not shown). Thus the presence of 0.1% (v/v) DMSO (vehicle) during treatment of cells did not change the equilibrium binding of NBMPR and that possible changes occurring as a result of treatment of cells with DMSO-dissolved PKC modulators would not be attributed to the vehicle. Fig. 10A shows results of mass law analysis and the determination of B_{max} of NBMPR binding in cells treated with vehicle or PMA, for 16 h. Treatment with 100 nM PMA for different intervals prior to NBMPR equilibrium binding studies demonstrated a time-dependent decline in B_{max} without change in K_D (Fig. 10A, Table 1). There was a significant difference between control results and those obtained in cells treated for 8 h or more with 100 nM PMA. Since prolonged treatment of cells with PMA results in down-regulation of PKC (6, 44), the observed decline in B_{max} during treatments for 8 h or more may be the result of PKC down-regulation. A plot of values of B_{max}, in cells treated with, as percent of the value in control (vehicle-treated) cells,

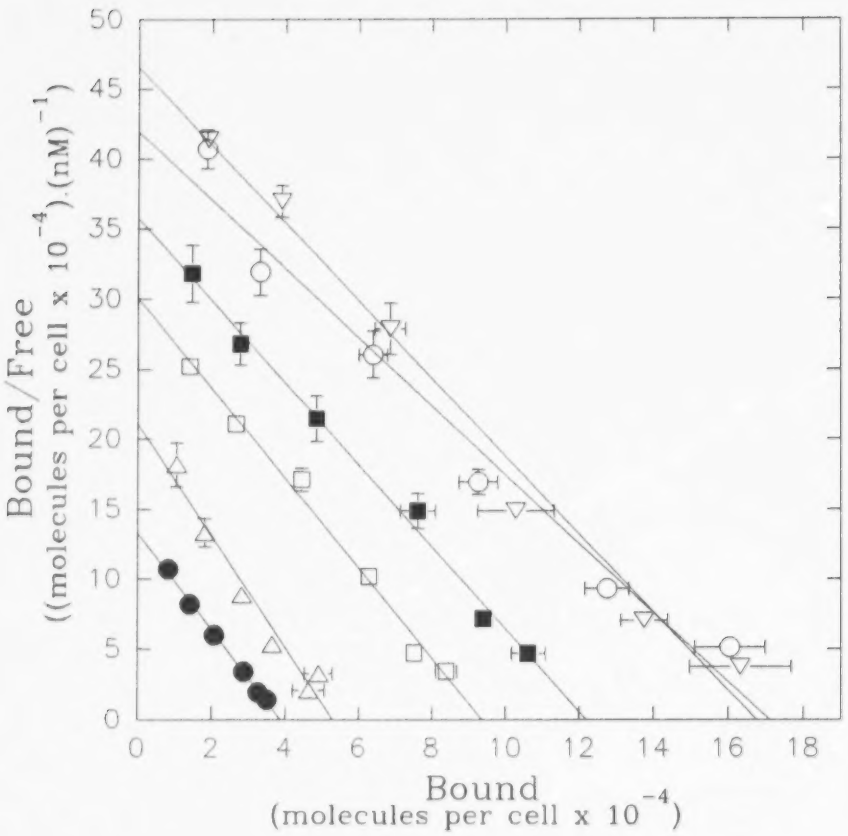

FIG. 10 (A). Scatchard analysis of the time-dependence of the effect of PMA on equilibrium binding of [^{3}H]NBMPR in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (O), or 100 nM PMA for 0.5 (∇), 2 (■), 8 (□), 16 (●) or 24 h (Δ). Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Assay mixtures contained 2 x 10^6 cells and graded concentrations of [^{3}H]NBMPR (0.125 -3 nM), with ("Non-specific binding") or without ("Total binding") 5 μM unlabeled NBMPR. "Bound", represents "Specific binding" obtained as the difference between "Total binding" and the "Non-specific binding", while "Free" is the unbound [^{3}H]NBMPR remaining in the medium. Each point is the mean ± S.E.M. of 12 experiments, each performed in triplicate.

	[^{3}H]NBMPR BINDING	
	B_{max} (molecules per cell x 10^{-4})	K_D (nM)
Control (0.1% vehicle)	17.4 ± 0.9	0.39
100 nM PMA, 0.5 h	16.9 ± 0.2	0.32
2 h	12.8 ± 0.6	0.41
8 h	9.4 ± 0.3*	0.40
16 h	3.9 ± 0.4*	0.31
24 h	5.2 ± 0.2*	0.28

Table 1. Levels of B_{max} for [^{3}H]NBMPR binding in CEM cells treated with 100 nM PMA for 0.5 to 24 h. Values of B_{max} and K_D were determined from Fig. 10 (A). *Significantly different from control values (P<0.05).

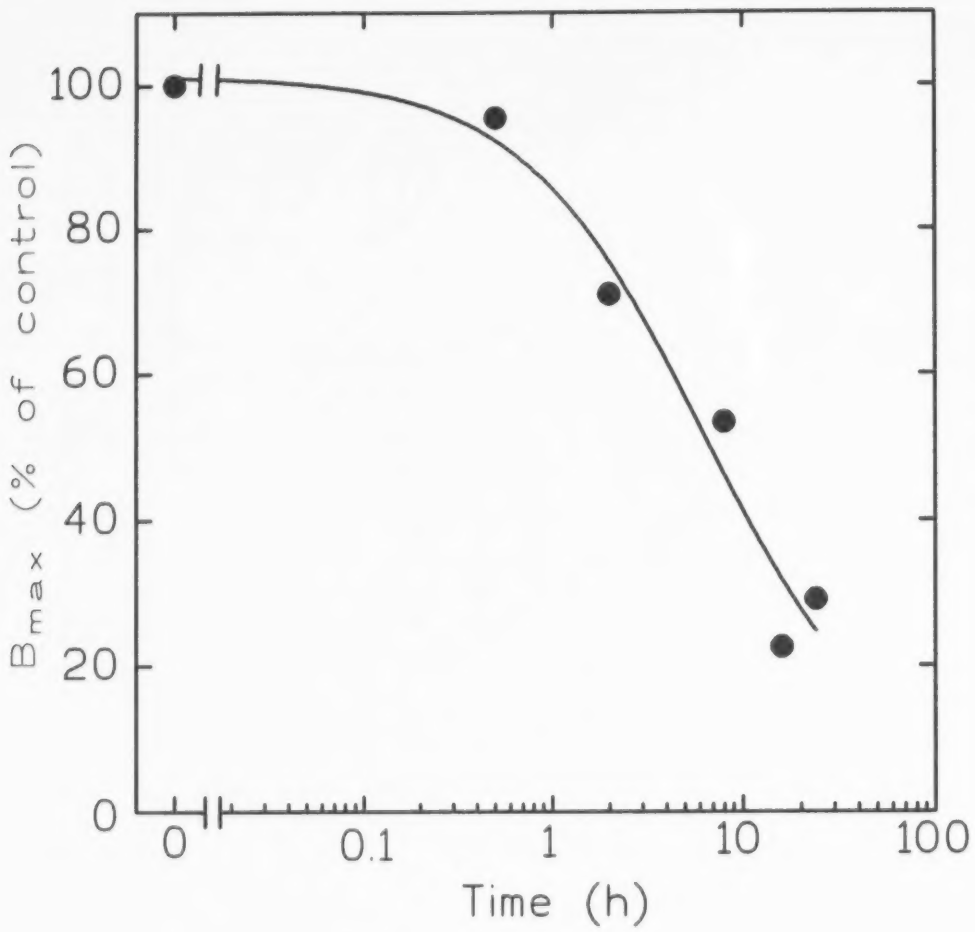

FIG. 10 (B). The effect of PMA treatment duration on B_{max} of [^{3}H]NBMPR binding in CEM cells. Values of B_{max} from the Scatchard analysis in Fig. 10 (A) are expressed as percent of control (vehicle-treated cells) value and plotted against time of exposure of cells to PMA.

against treatment period (Fig. 10B) indicates that after about 5.4 h of treatment of cells with 100 nM PMA, the B_{max} for NBMPR binding declined to 50% of the control value. The value of B_{max} in 24-h treated cells was not significantly different from the value obtained in 16-hr PMA-treated cells. A treatment period of 16 h was therefore chosen for investigation of the effects of graded concentrations of PMA on NBMPR equilibrium binding in CEM cells.

Incubation of CEM cells for 16 h with PMA, over a 1 to 200 nM range prior to NBMPR equilibrium binding studies, caused a concentration-dependent reduction in B_{max} for NBMPR binding without changes in K_D (Fig. 10C). Treatment for 16 h with PMA resulted in a decrease of B_{max} values with no change in K_D (Fig. 10C, Table 2). These data are plotted as B_{max} as percent of control against concentration of PMA in Fig. 10D. The IC_{50} (the concentration of PMA, which resulted in 50% reduction in B_{max} for NBMPR binding) obtained was 9.2 nM. Treatment of cells with 200 nM PMA did not result in a significant change in B_{max} from the value observed in cells treated with 100 nM PMA.

3.3.2. Effect of phorbol 12,13-dibutyrate (PDBu) and 4α-phorbol 12,13-didecanoate (4α-PDD) on equilibrium binding of NBMPR in CEM cells

To extend the observation of reduced B_{max} for NBMPR binding made in PMA-treated cells, NBMPR equilibrium binding studies were carried out in CEM cells previously treated for 16 h with 100 nM phorbol 12,13-dibutyrate (PDBu), another active phorbol ester. As in PMA-treated cells, the value of B_{max} for NBMPR binding was reduced from 17. 4 x 10^4 molecules/cell (K_D, 0.39 nM), in vehicle-treated cells, to 7.1 x 10^4 molecules/cell in PDBu-treated cells, without any significant change in K_D, 0.45 nM (Fig. 10E). In contrast, B_{max} for NBMPR binding (16.1 x 10^4 molecules/cell; K_D, 0.36 nM) obtained in CEM cells treated for 16 h with 100 nM 4α-phorbol 12, 13-didecanoate

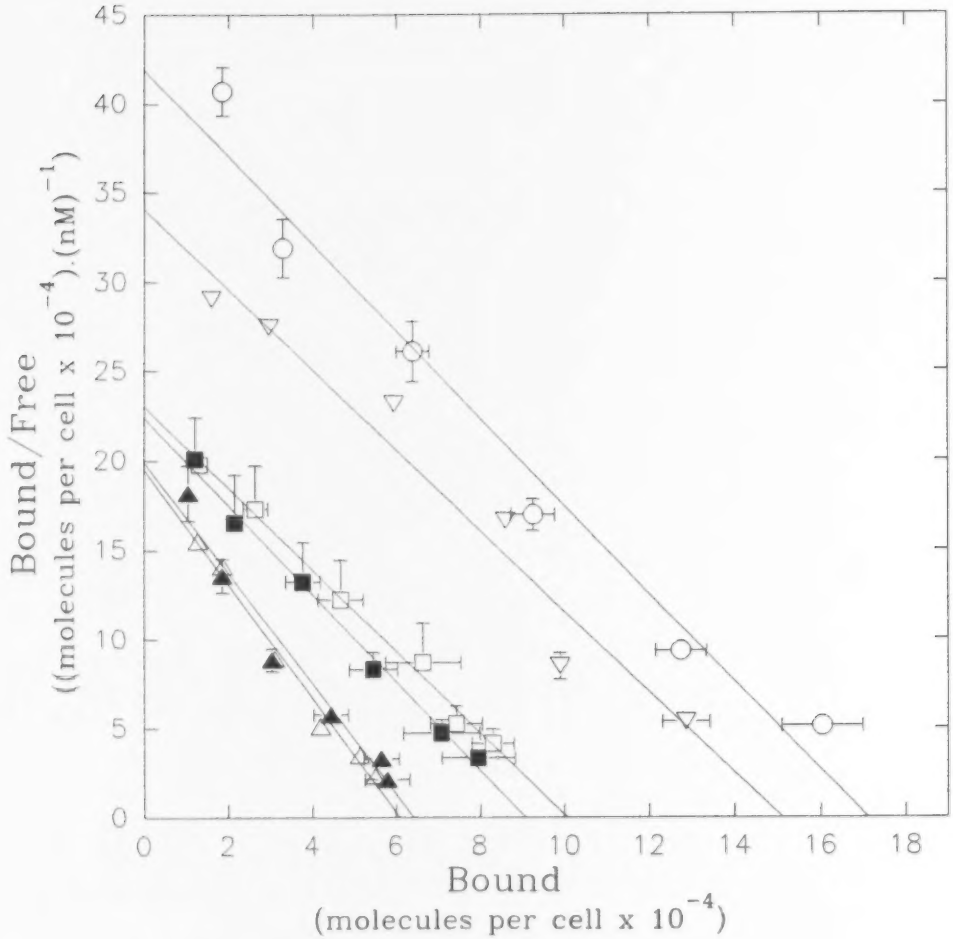

FIG. 10 (C). Scatchard analysis of the concentration-dependence of the effect of PMA on equilibrium binding of [^{3}H]NBMPR in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (O), or 1 (∇), 10 (□), 50 (■), 100 (▲) or 200 nM (Δ) PMA for 16 h. Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 12 experiments, each performed in triplicate.

	[^{3}H]NBMPR BINDING	
	B_{max} (molecules per cell x 10^{-4})	K_D (nM)
Control (0.1% vehicle)	17.4 ± 0.9	0.39
[PMA] (nM) 1	15.2 ± 0.9	0.43
10	10.1 ± 0.2*	0.44
50	9.1 ± 0.5*	0.41
100	6.4 ± 0.4*	0.33
200	6.0 ± 0. 5*	0.31

Table 2. Levels of B_{max} for [^{3}H]NBMPR binding in CEM cells treated with 1-200 nM PMA for 16 h. Values of B_{max} and K_D were determined from Fig. 10 (C). *Significantly different from control values (P<0.05).

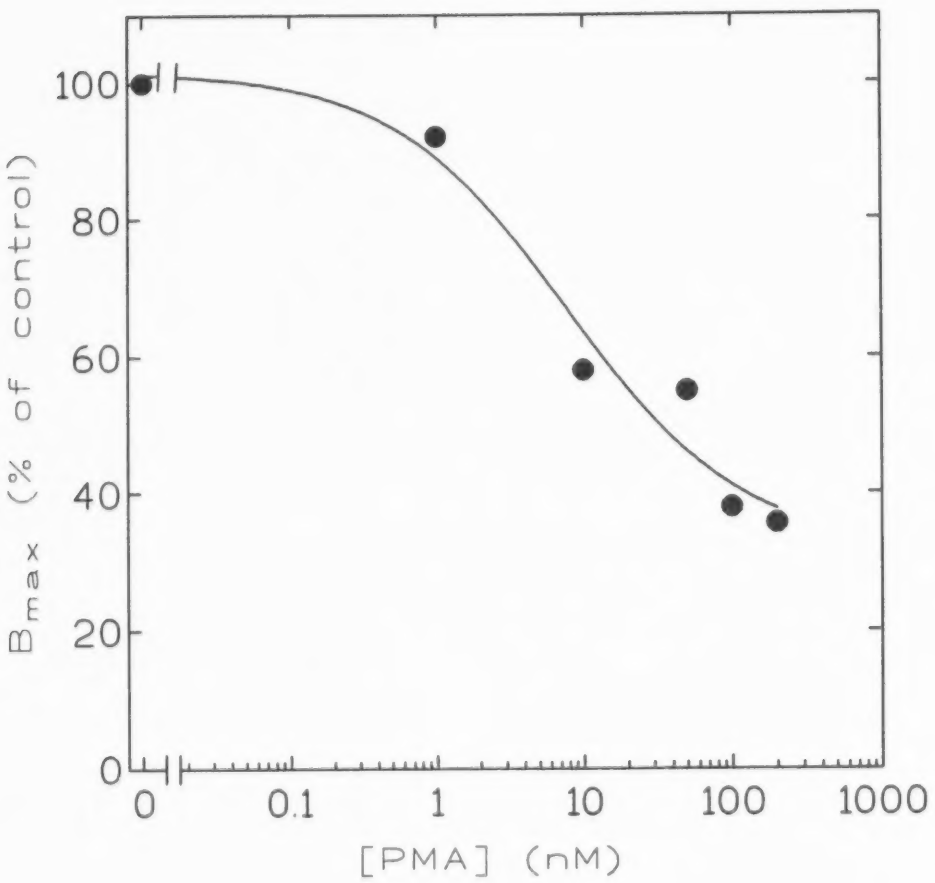

FIG. 10 (D). The effect of increasing concentration of PMA on B_{max} for [^{3}H]NBMPR binding in CEM cells. Values of B_{max} from the Scatchard analysis in Fig. 10 (C), are expressed as percent of control (vehicle-treated cells) value and plotted against concentration of PMA.

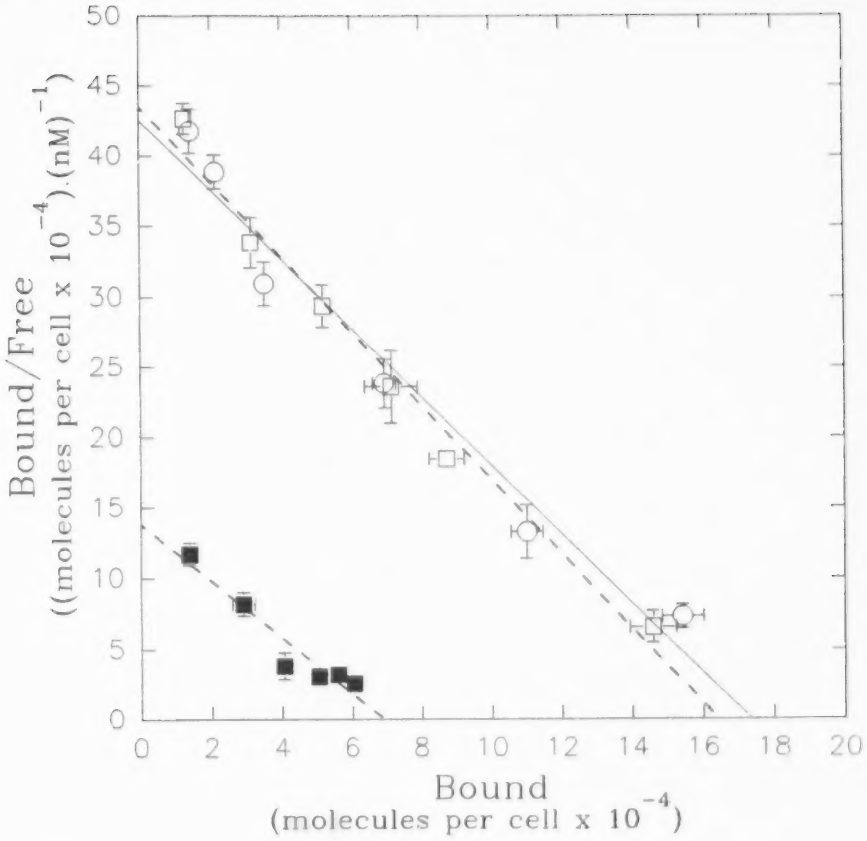

FIG. 10 (E). Scatchard analysis of the effect of treatment of CEM cells with 4α-PDD or PDBu on equilibrium binding of [^{3}H]NBMPR. Cells were treated with 0.1% (v/v) DMSO (vehicle) (O), or 100 nM of 4α-PDD (□) or PDBu (■) for 16 h. Afterwards, cells were washed, and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of at least 4 experiments, each performed in triplicate.

(4α-PDD), an inactive phorbol ester which does not activate PKC (210) and cannot cause down-regulation of the kinase, was not significantly different from B_{max} obtained in vehicle-treated cells (Fig. 10E). This observation supports the idea that the decrease in B_{max} for NBMPR binding in cells treated with active phorbol esters, PMA or PDBu, is attributed to the actions of these agents on PKC.

3.3.3. Effects of Staurosporine (STA) and bisindolylmaleimide, GF 109203X (BIM) on equilibrium binding of NBMPR in CEM cells

To assess the effect of inhibition of PKC on the cellular abundance of NBMPR binding sites, equilibrium binding studies of NBMPR were conducted in CEM cells pretreated with STA. Fig. 11A shows that in cells treated with 100 nM STA for 0.5 to 24 h, there was a decline in B_{max} for NBMPR binding with no significant change in K_D. The extent of the decline in B_{max} was dependent on the length of incubation of cells with the PKC inhibitor. Values of B_{max} and K_D in STA-treated cells are shown in Table 3. In Fig. 11B, the data from Fig. 11A have been plotted as B_{max} (percent of control) against treatment period. The treatment period that resulted in 50% decline of B_{max} was determined to be about 4.5 h.

Treatment of CEM cells with STA caused a concentration-dependent decrease in B_{max} for NBMPR binding with no significant change in K_D. In cells treated for 16 h with STA, B_{max} for NBMPR binding decreased with no change in K_D (Fig. 11C). Values of B_{max} and K_D in STA-treated cells are shown in Table 4. In Fig. 11D, the data of Fig. 11C have been plotted as B_{max} (percent of control) against STA concentration. It appears that the curve nearly levels off at STA concentration about 30 nM (Fig. 11D), and that treatment with high concentrations of STA, such as 100 or 200 nM, did not decrease B_{max} to below 40% of control.

Exposure of cells with 2.0 μM bisindolylmaleimide (GF 109203X, BIM), another PKC inhibitor, produced a small change in B_{max}, from 17.98×10^4 molecules/cell, K_D,

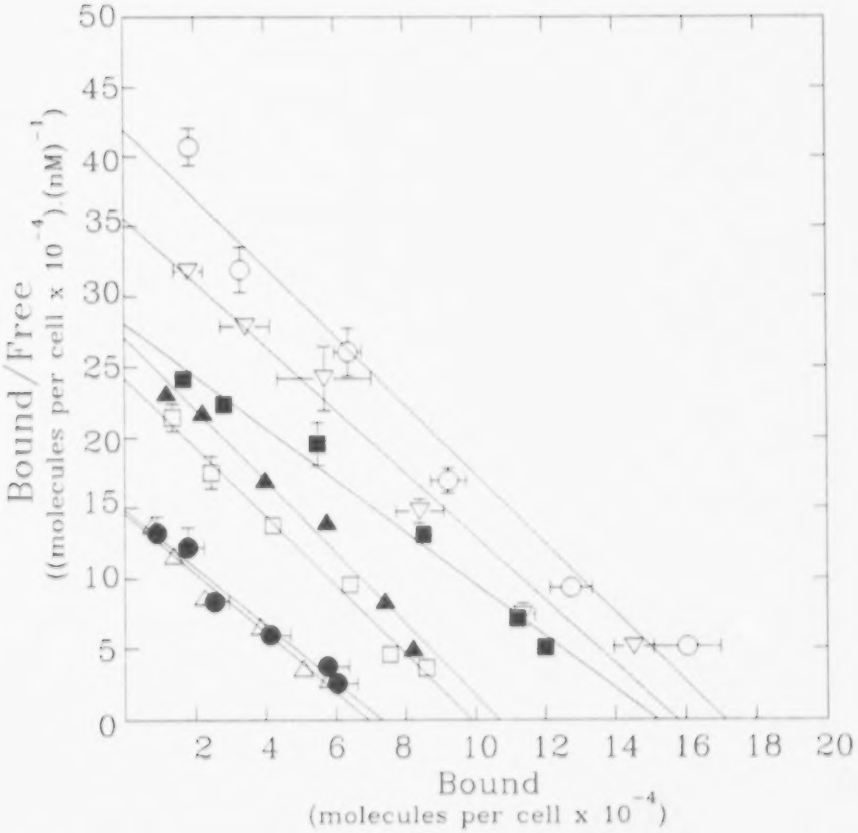

FIG. 11 (A). Scatchard analysis of the time-dependence of the effect of STA on equilibrium binding of [^{3}H]NBMPR in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (O), or 100 nM STA for 0.5 (∇), 2 (■), 6 (▲), 8 (□), 16 (●) or 24 h (Δ). Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 12 experiments, each performed in triplicate.

	[^{3}H]NBMPR BINDING	
	B_{max} (molecules per cell x 10^{-4})	K_D (nM)
Control (0.1% vehicle)	17.4 ± 0.9	0.39
100 nM STA, 0.5 h	15.8 ± 0.3	0.41
2 h	15.2 ± 0.2	0.48
6 h	10.8 ± 0.2*	0.39
8 h	9.8 ± 0.5*	0.39
16 h	6.8 ± 0.3*	0.45
24 h	7.3 ± 0.5*	0.47

Table 3. Levels of B_{max} for [^{3}H]NBMPR binding in CEM cells treated with 100 nM STA for 0.5 to 24 h. Values of B_{max} and K_D were determined from Fig. 11 (A). *Significantly different from control values (P<0.05).

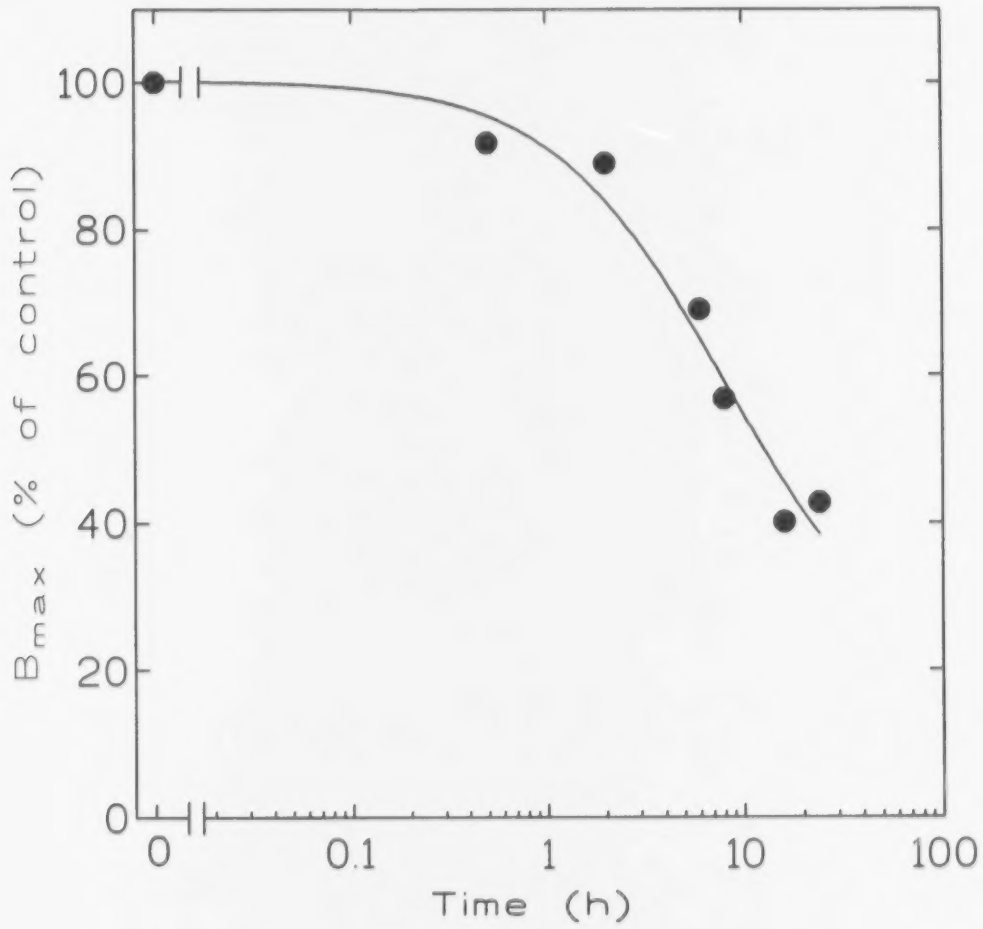

FIG. 11 (B). The effect of STA treatment duration on B_{max} for [^{3}H]NBMPR binding in CEM cells . Values of B_{max} from the Scatchard analysis in Fig. 11 (A) are expressed as percent of control (vehicle-treated cells) value and plotted against time of exposure of cells to STA.

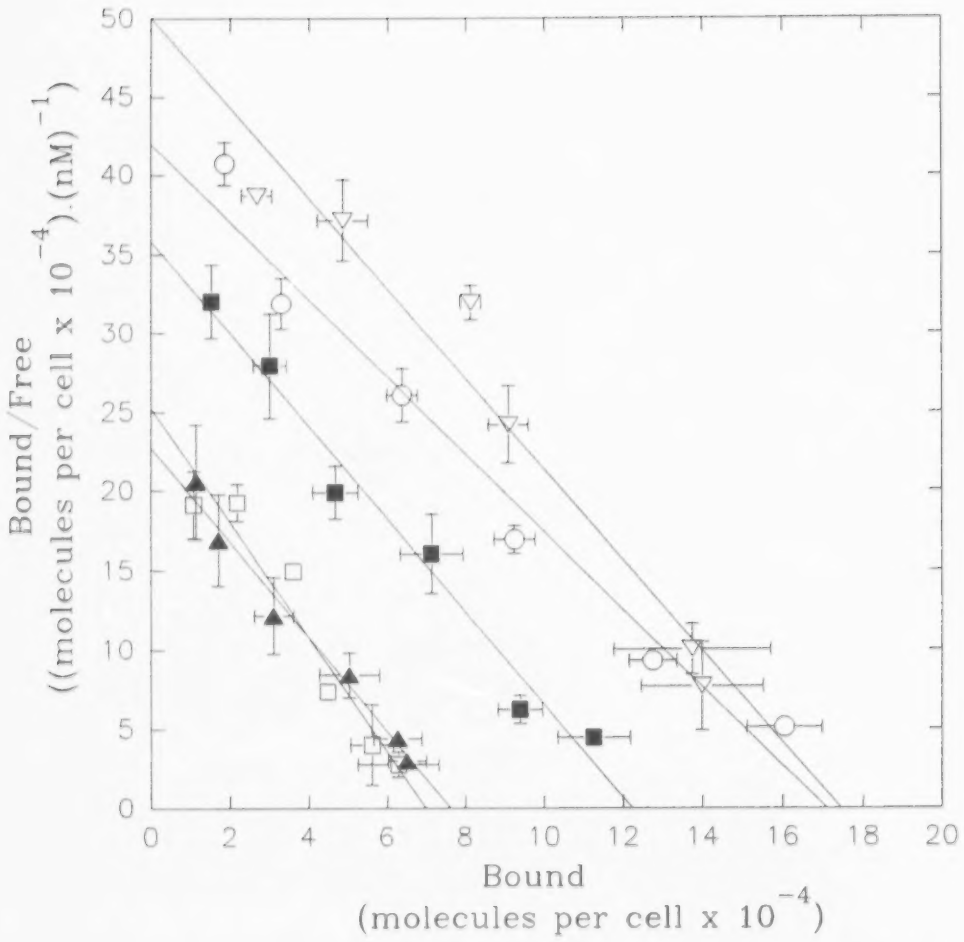

FIG. 11 (C). Scatchard analysis of the concentration-dependence of STA effect on equilibrium binding of [^{3}H]NBMPR in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (O), or 1 (▽), 10 (■), 100 (▲) or 200 nM (□) STA for 16 h. Subsequently, cells were washed and resuspended in RPMIt. Equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 12 experiments, each performed in triplicate.

	[^{3}H]NBMPR BINDING	
	B_{max} (molecules per cell x 10^{-4})	K_D (nM)
Control (0.1% vehicle)	17.4 ± 0.9	0.39
[STA] (nM) 1	17.2 ± 0.6	0.41
10	12.1 ± 0.4*	0.34
100	7.8 ± 0.3*	0.33
200	6.9 ± 0.5*	0.29

Table 4. Levels of B_{max} for [^{3}H]NBMPR binding in CEM cells treated with 1-200 nM STA for 16 h. Values of B_{max} and K_D were determined from Fig. 11 (C). *Significantly different from control values ($P<0.05$).

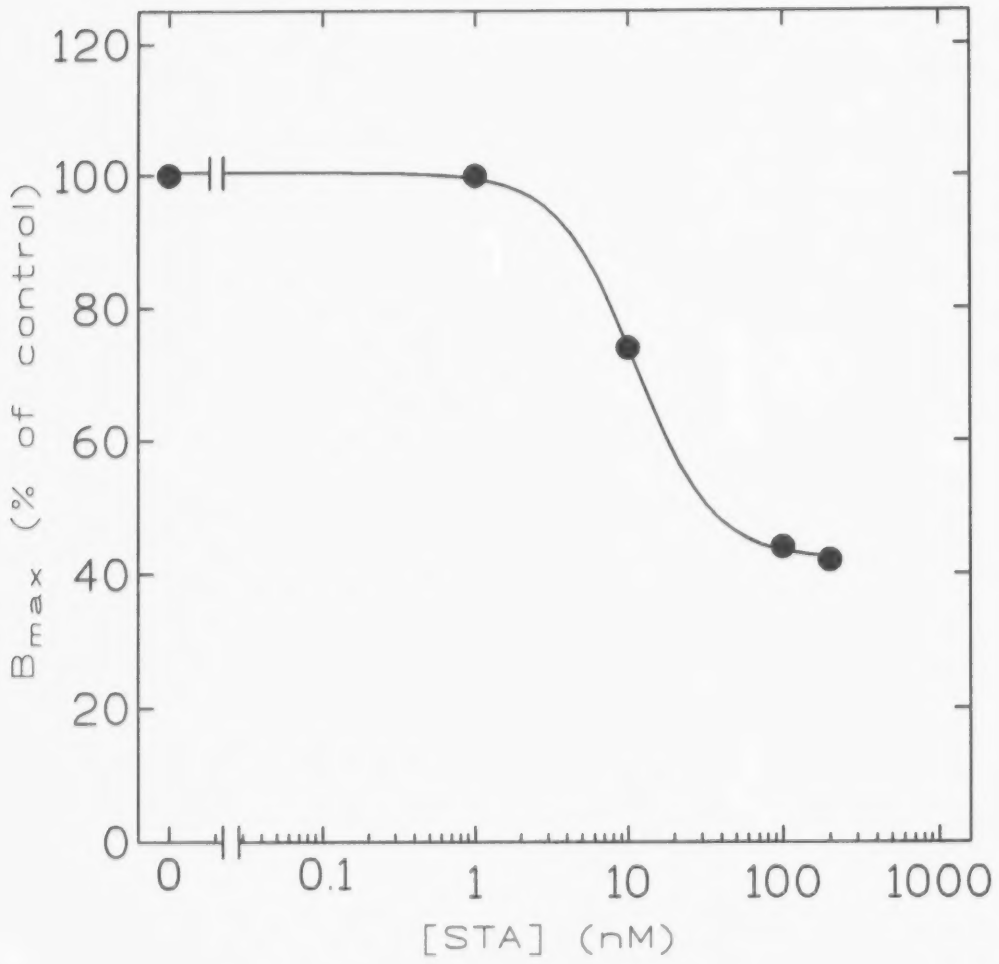

FIG. 11 (D). The effect of increasing concentration of STA on B_{max} for [^{3}H]NBMPR binding in CEM cells. Values of B_{max} from the Scatchard analysis in Fig. 11 (C) are expressed as percent of control (vehicle-treated cells) value and plotted against concentration of STA.

0.31 nM, to 14.8 x 10^4 molecules/cell, K_D, 0.30 nM (Fig. 11E). This change was not found to be significant ($p>0.05$). Thus, BIM (2 μM) induced only a slight decline in B_{max} for NBMPR binding. Treatment of cells with higher concentrations of BIM was not carried out because concentrations above 4 μM were found to cause a rapid decline in cell numbers in a preliminary cell proliferation study (result not shown). BIM is a less potent inhibitor of PKC than STA (211). Furthermore, the ability of the maleimide drugs to bind irreversibly to sulfhydryl groups on membrane proteins (212, 213) may have prevented attaining sufficient concentrations of the drug in cells to inhibit PKC, and might account for the lack of BIM effect on NBMPR binding in CEM cells.

3.3.4. Effect of treatment of CEM cells with other agents on equilibrium binding of NBMPR

Although it was the most potent inhibitor of PKC in these studies, STA is nevertheless non-specific (74). For example, STA inhibited cAMP-dependent protein kinase (PKA) and $p60^{v\text{-}src}$ tyrosine protein kinase with comparable IC_{50} values of 8 nM and 6 nM, respectively (compare with IC_{50} for PKC inhibition, 3 nM) (74). Thus, to support the idea that the STA-mediated decrease in B_{max} for NBMPR binding in STA-treated cells in our studies is via PKC inhibition, we pretreated cells for 16 h with inhibitors more specific for PKA, cGMP-dependent protein kinase, and tyrosine kinase. Subsequently, measurements of NBMPR binding were carried out in drug-treated cells. Results in Table 5 show that exposure of cells to HA1004, an inhibitor of PKA and cGMP-dependent protein kinase, or genistein, a tyrosine kinase inhibitor, did not induce any change in binding of NBMPR to high affinity sites. These results suggest that neither PKA, cGMP-dependent protein kinase nor tyrosine kinase was involved in the mediation of the STA-induced decline in B_{max} for NBMPR binding observed in our studies. As was shown earlier in this study (Fig. 10E), treatment of cells for 16 h with an inactive phorbol

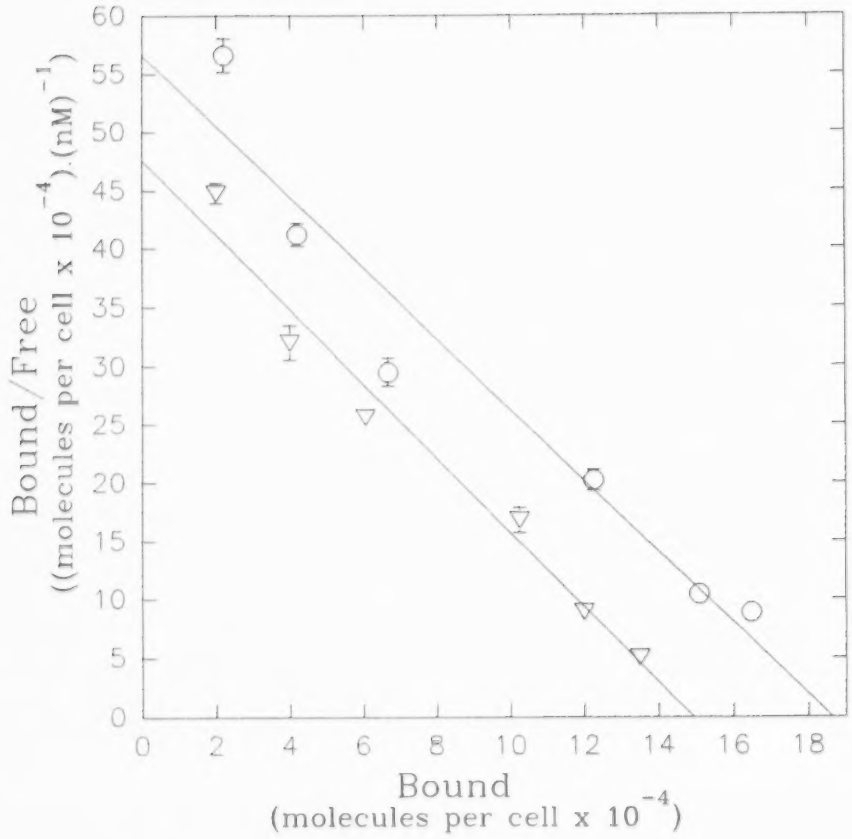

FIG. 11 (E). Scatchard analysis of the effect of treatment of CEM cells with BIM on equilibrium binding of [^{3}H]NBMPR. Cells were exposed to 0.1% (v/v) DMSO (vehicle) (O) or 2 μM BIM (∇) for 16 h. Subsequently, cells were washed and resuspended in RPMIt. Equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Results are the mean ± S.E.M. of 6 replicate measurements in 2 separate experiments.

	Specific binding of [^{3}H]NBMPR	
	molecules per cell x 10^{-4}	% of control
Control (0.1% vehicle)	15.6 ± 0.9	100
100 nM PMA	6.0 ± 0.2	39*
100 nM STA	5.2 ± 0.3	33*
100 nM 4α-PDD	14.5 ± 0.4	93
50 μM genistein	15.5 ± 0.1	100
40 μM H7	8.5 ± 0.2	55*
40 μM HA1004	15.5 ± 0.4	100

Table 5. Effects of various agents on the B_{max} for [^{3}H]NBMPR binding in CEM cells. Cells were treated with the agents at the concentrations indicated for 16 h. Following this, cells were washed and resuspended in RPMIt and measurements of equilibrium binding of [^{3}H]NBMPR was carried out as described in "Materials and Methods". In these experiments, a single high concentration (4.0 nM) [^{3}H]NBMPR was used to determine B_{max}. Each value is the mean ± S.E.M. of 6 replicate measurements in two separate experiments. *Significantly different from control values (P<0.05).

ester, 4α-PDD, did not affect equilibrium binding of NBMPR in drug-treated cells (Table 5). In contrast, treatment of cells for 16 h with another non-selective inhibitor of PKC, H7, reduced site-specific binding of [^{3}H]NBMPR from 15.6 to 8.5 x 10^4 molecules/cell, (54.5% of control) (Table 5). H7 is reported to inhibit cGMP-dependent protein kinase, PKA, myosin light chain kinase and PKC, with IC_{50} values of 6 μM, 3 μM, 97 μM, and 6 μM respectively (74). However, the lack of effect on NBMPR binding in cells treated with inhibitors of PKA or cGMP-dependent kinase suggests that it was unlikely that H7-mediated change in binding of NBMPR in this study involved PKA or cGMP-dependent protein kinase. The concentration of H7 used in these experiments, 40 μM, was less than half of the IC_{50} value for inhibitor of myosin light chain kinase, further arguing in favor of inhibition of PKC by H7 rather than myosin light chain kinase as the underlying cause of the decrease in NBMPR binding. Also obvious from Table 5 was the effect of preincubation of cells for 16 h with PMA or STA, which resulted in decline in site-specific binding of NBMPR in these experiments (Figs. 10A-11D).

3.3.5 Effect of treatment of CEM cells with PMA on inward fluxes of Ado

Only NBMPR-sensitive (*es*) nucleoside transporters are expressed in CEM cells (102). In order to determine whether reduction of B_{max} for NBMPR binding in PMA-treated cells was associated with changes in nucleoside transport activity in these cells, the uptake of [^{3}H]Ado was measured in PMA-treated cells.

The uptake of 1.0 μM [^{3}H]Ado was measured in cells treated for 16-h, with 0.1% (v/v) DMSO (vehicle), or in cells treated with 100 nM PMA, for 0.5 to 24 h. During the first 9 s of Ado uptake, progress curves were linear, and inward fluxes were calculated as slopes of lines fitted to data by linear regression, which were initial rates of uptake.

The uptake of [^{3}H]Ado in the presence and in absence of 5 μM unlabeled NBMPR, in cells treated or untreated with 0.1% (v/v) DMSO vehicle was determined

(Fig. 12Ai). In the presence of NBMPR uptake of [^{3}H]Ado was negligible. Treatment of cells with the vehicle alone did not alter the rate of Ado influx, (Fig. 12Ai). Fig. 12Aii shows that treatment of cells with 100 nM PMA reduced rate of Ado influx into cells; this decrease was dependent on the length of the incubation time of cells with PMA. Rates of Ado influx measured in control and PMA-treated cells are shown in Table 6. A plot of the data of Fig. 12Aii as rate of Ado influx (percent of control) against period of treatment of cells with PMA showed that after 4.5 h of treatment of cells with 100 nM PMA, Ado influx rate was reduced to 50% of the control value (Fig. 12B). The treatment of cells with PMA for over 16 h did not result in further decline of Ado influx rates to the zero time value even though during this period of treatment, PKC activity was reduced to almost zero (see Fig. 9). (It is therefore likely that PKC down-regulation is the underlying mechanism for the decline in the rate of Ado influx and B_{max} in PMA-treated cells). The observation that Ado influx in phorbol ester-treated cells was not completely abolished correlates with the similar observation made in equilibrium binding studies of NBMPR in PMA-treated cells.

Pretreatment of CEM cells with PMA also showed a concentration-dependent decrease in rate of Ado influx into cells (Fig. 12C). Rates in control (vehicle-treated) and in PMA-treated cells are shown in Table 7. Fig. 12D is a plot of the data from Fig. 12C as Ado influx rate (percent of control) against concentration of PMA. The IC_{50} value obtained from this curve was 11 nM. It seems from the result that treatment of cells with PMA at concentrations higher than 100 nM did not elicit any further decrease in Ado influx rate.

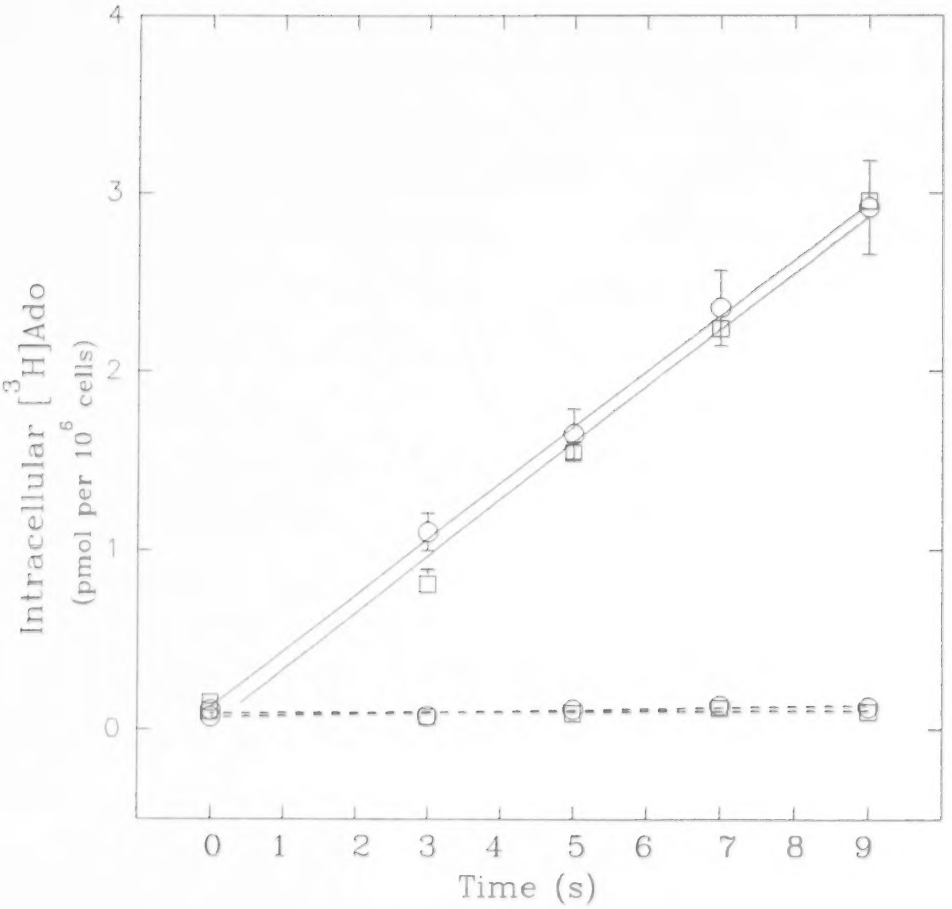

FIG. 12 (Ai). Time-course of [^{3}H]Ado influx in CEM cells treated with DMSO (vehicle). Cells were treated with (□) or without 0.1% (v/v) DMSO (vehicle) (O) for 16 h. Following treatment, cells were washed and resuspended in RPMIt. Uptake of [^{3}H]Ado was measured (solid lines) as described under "Materials and Methods". Measurements of uptake of [^{3}H]Ado in the presence of 5 μM NBMPR were also carried out in each cell suspension (dashed lines). Each point is the mean ± S.E.M. of at least 4 separate experiments, each performed in triplicate.

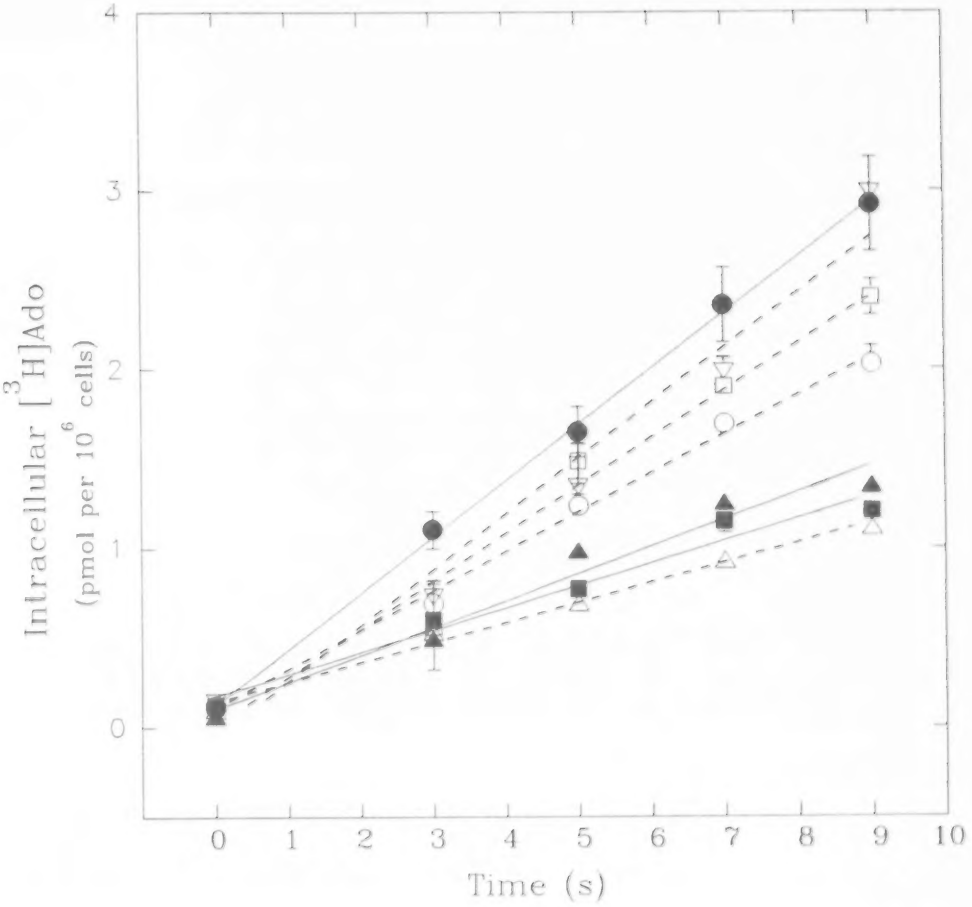

FIG. 12 (Aii). Time-dependence of PMA effect on the influx of 1 μM [^{3}H]Ado in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM PMA for 0.5 (▽), 2 (□), 4 (O), 8 (▲), 16 (Δ) or 24 h (■). Subsequently, cells were washed and resuspended in RPMIt. Uptake of [^{3}H]Ado was measured as described in "Materials and Methods". Time-zero values were determined in the presence of 5 μM NBMPR. Each point is the mean ± S.E.M. of 10 separate experiments, each performed in triplicate.

	1.0 μM [^{3}H]Ado influx rate (pmol per s per 10^6 cells)
Control (0.1% vehicle)	0.31
100 nM PMA, 0.5 h	0.31
2 h	0.24
4 h	0.23
8 h	0.14*
16 h	0.11*
24 h	0.13*

Table 6. Rates of [^{3}H]Ado influx in CEM cells treated with 100 nM PMA for 0.5 to 24 h. Rates were calculated from progress curves in Fig. 12 (Aii). *Significantly different from control values (P<0.05).

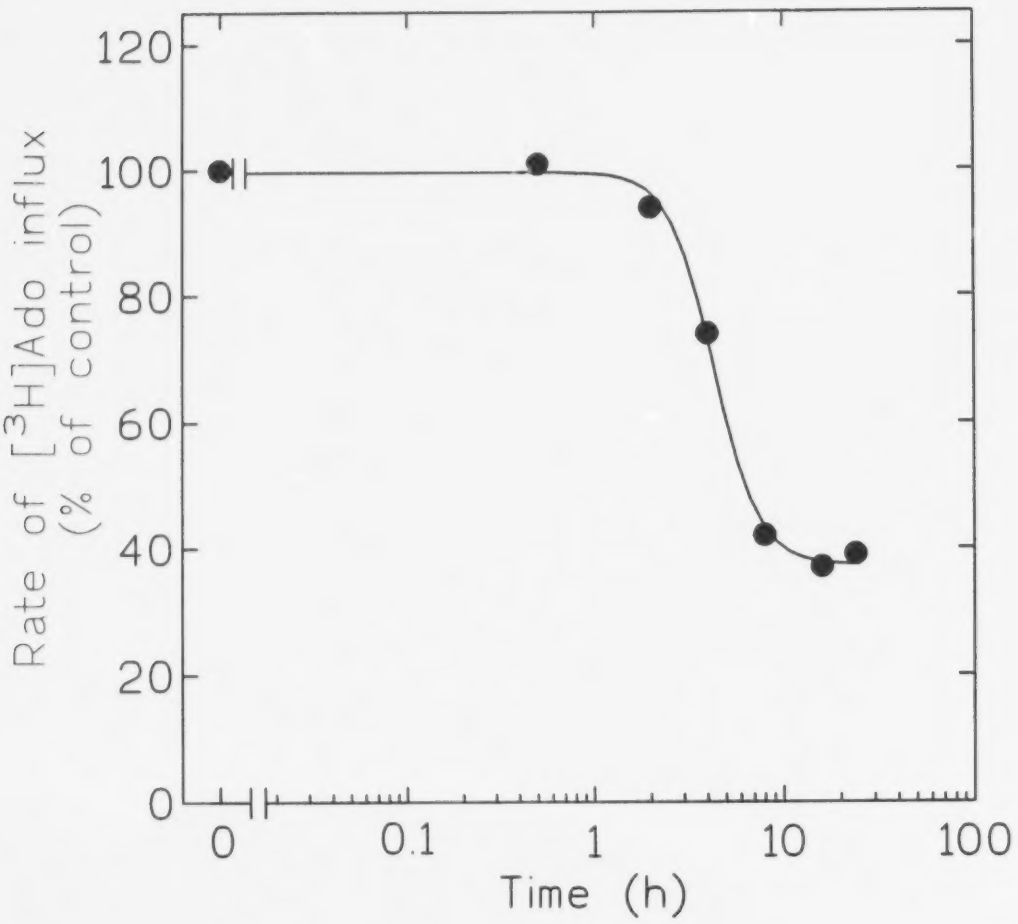

FIG. 12 (B). The effect of PMA treatment duration on the influx rate of 1 μM [^{3}H]Ado in CEM cells. Rates of influx of Ado, obtained as the slopes of progress curves in Fig. 12 (Aii), are expressed as percent of rate in control (vehicle-treated) cells, and plotted against duration of PMA treatment. The inward flux of [^{3}H]Ado in control cells was 0.31 pmol per s per 10^6 cells.

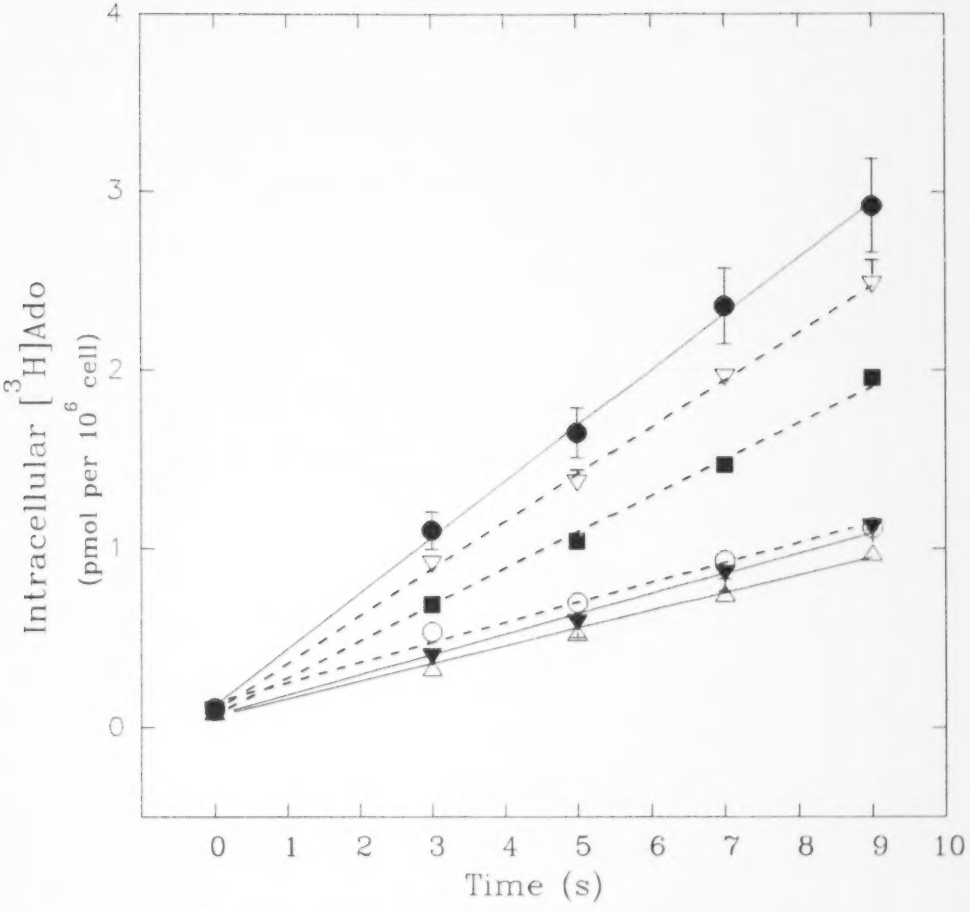

FIG. 12 (C). Concentration-dependence of PMA effect on the influx of 1 μM [^{3}H]Ado in CEM cells. Cells were treated for 16 h with 0.1% (v/v) DMSO (vehicle) (●), or 1 (▽), 10 (■), 100 (O), 200 (▼) or 500 nM (Δ) PMA. Subsequently, cells were washed and resuspended in RPMIt. Uptake of [^{3}H]Ado was measured as described under "Materials and Methods". Each point is the mean ± S.E.M. of 10 separate experiments, performed in triplicate.

	1.0 μM [^{3}H]Ado influx rate (pmol per s per 10^6 cells)
Control (0.1% vehicle)	0.31
[PMA] (nM) 1	0.28
10	0.21
100	0.13*
200	0.12*
500	0.10*

Table 7. Rates of [^{3}H]Ado influx in CEM cells treated for 16 h with 1-500 nM PMA. Rates were calculated from progress curves in Fig. 12 (C). *Significantly different from control values (P<0.05).

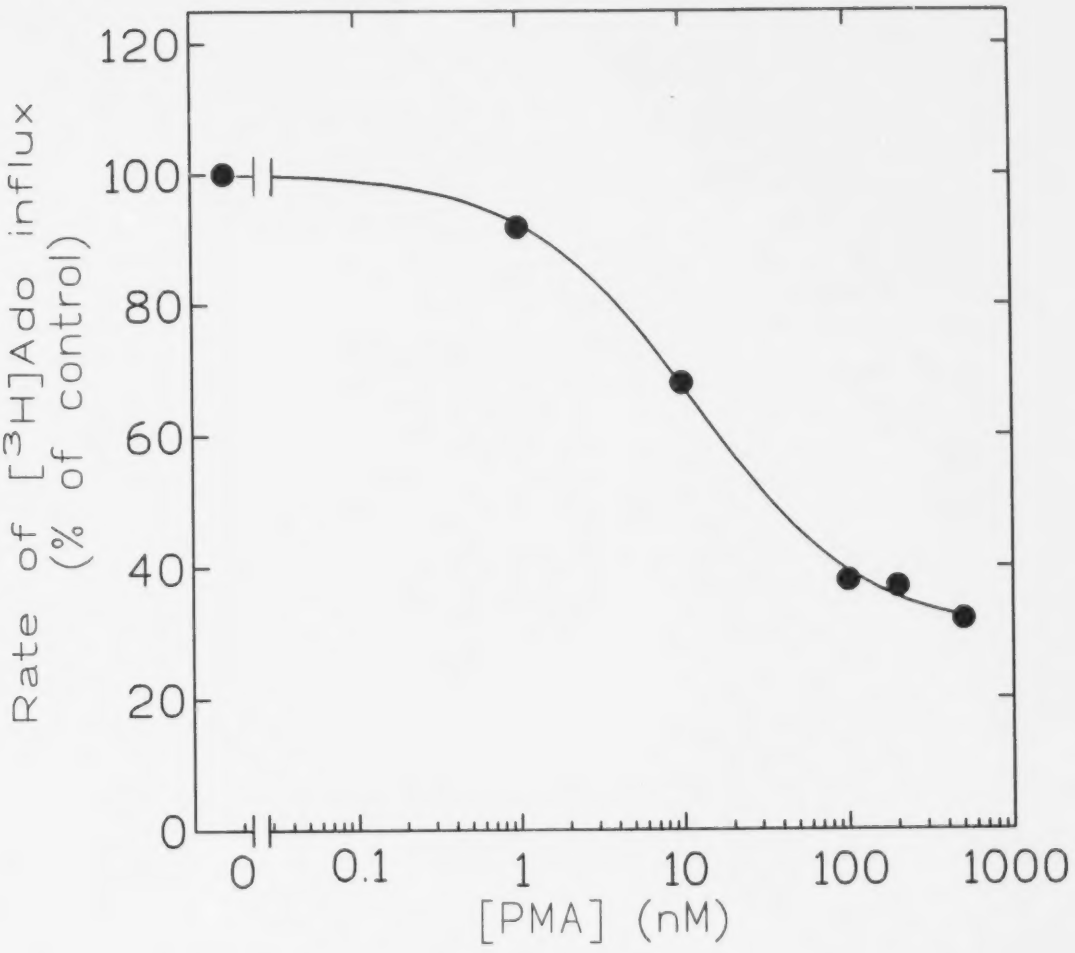

FIG. 12 (D). The effect of increasing concentration of PMA on the rate of 1 μM [^{3}H]Ado influx in CEM cells. Rates of influx obtained as the slopes of progress curves in Fig. 12 (C), are expressed as percent of rate in control (vehicle-treated) cells and plotted against concentration of PMA. The inward flux of [^{3}H]Ado in control cells was 0.31 pmol per s per 10^6 cells.

This is demonstrated as the nearly horizontal portion of the curve in Fig. 12D, at concentrations of PMA that were 100 nM and higher.

3.3.6. Effect of treatment of CEM cells with STA on inward fluxes of Ado

Uptake of 1 μM [^{3}H]Ado was measured in cells treated with STA to determine whether reduction of B_{max} for NBMPR binding in STA-treated cells was associated with changes in nucleoside transport activity. Results in Fig. 13A show that rate of Ado influx in STA-treated cells was reduced in a treatment time-dependent manner. Rates of influx in control (vehicle-treated) and in STA-treated cells are shown in Table 8. The effect of treatment of cells with STA was significant after a prolonged (8 h or more) of incubation of cells with the PKC inhibitor. When the data of Fig. 13A are presented as percent of Ado influx rate in control cells and plotted against treatment period, the length of time of treatment of cells with STA that caused 50% reduction in rate of Ado influx was determined to be 5 h (Fig. 13B). Also obvious from the graph in Fig. 13B is the fact that rate of Ado influx in STA-treated cells was reduced to only about 40% of rate in control cells by the 24 h treatment period.

Preincubation of CEM cells with STA also resulted in concentration-dependent decrease in the rate of Ado influx into STA-treated cells (Fig. 13C). The rates of influx are represented in Table 9. The decline in rates of Ado influx occurred following incubation of cells with STA at concentrations that are known to inhibit PKC suggesting that a STA-PKC interaction underlies the decline in NT activity observed in STA-treated cells. In Fig. 13D, the data of Fig. 13C have been plotted as Ado influx rates (percent of control) against concentration of STA. The rate of Ado influx continued to decline to 20% of value in control (vehicle-treated) cells at STA concentration of 400 nM (Fig. 13D).

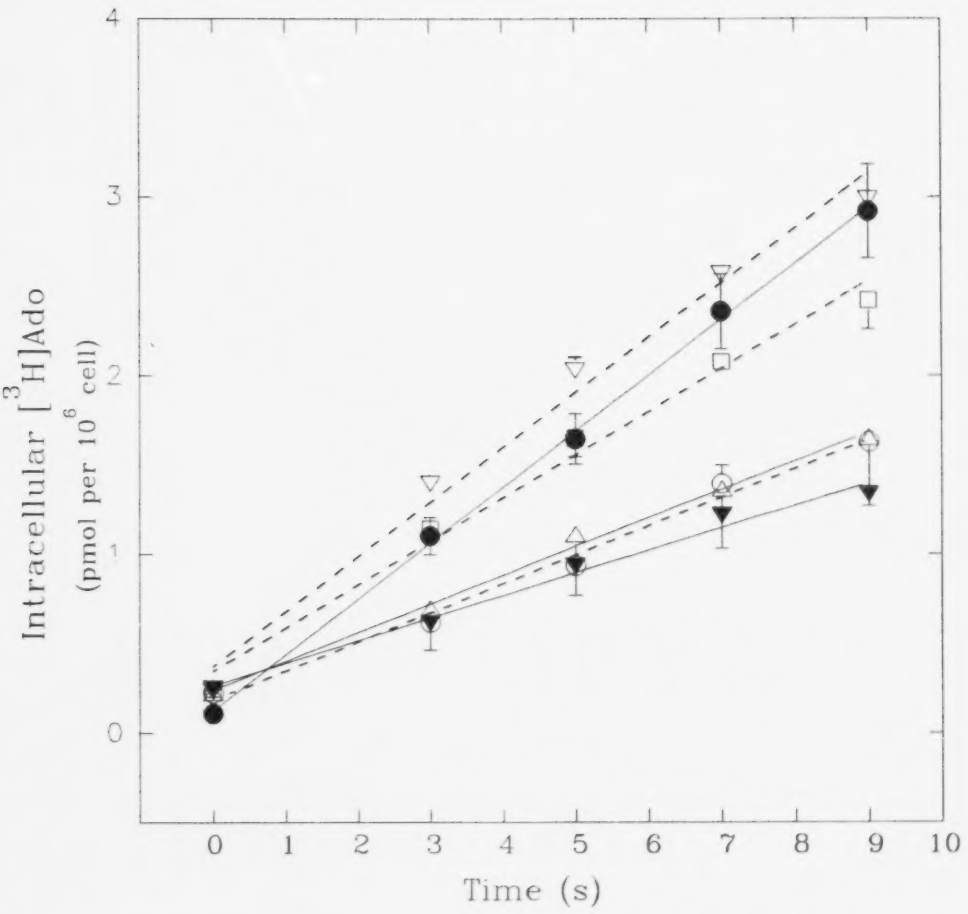

FIG. 13 (A). Time-dependence of STA effect on the influx of 1 μM [^{3}H]Ado in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM STA for 0.5 (▽), 4 (□), 8 (O), 16 (Δ) or 24 h (▼). After these times, cells were washed and resuspended in RPMIt. Uptake of [^{3}H]Ado was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 10 separate experiments, each performed in triplicate.

	1.0 μM [^{3}H]Ado influx rate (pmol per s per 10^6 cells)
Control (0.1% vehicle)	0.31
100 nM STA, 0.5 h	0.30
4 h	0.25
8 h	0.16*
16 h	0.15*
24 h	0.11*

Table 8. Rates of [^{3}H]Ado influx in CEM cells treated with 100 nM STA for 0.5 to 24 h. Rates were calculated from progress curves in Fig. 13 (A). *Significantly different from control values (P<0.05).

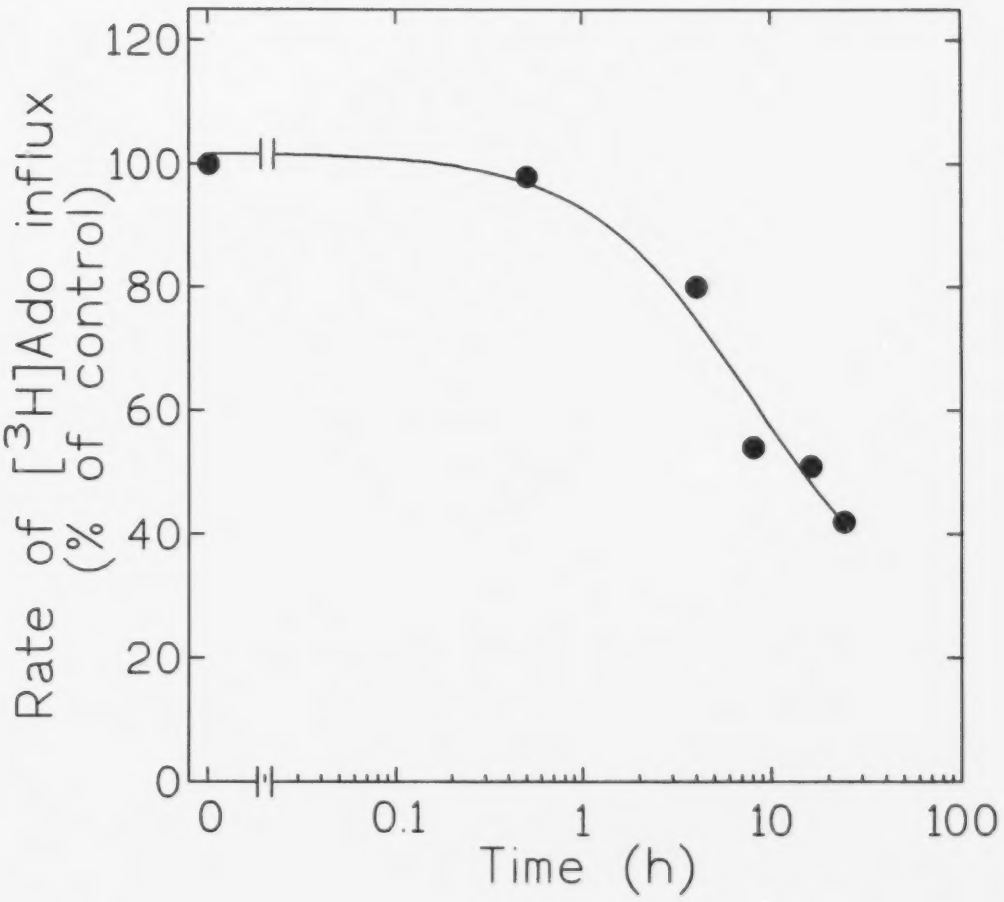

FIG. 13 (B). The effect of STA treatment duration on the influx of 1 μM [^{3}H]Ado in CEM cells. Rates of influx obtained as the slopes from progress curves in Fig. 13 (A), are expressed as percent of rate in control (vehicle-treated) cells, and plotted against duration of STA treatment. The inward flux in control cells was 0.31 pmol per s per 10^6 cells.

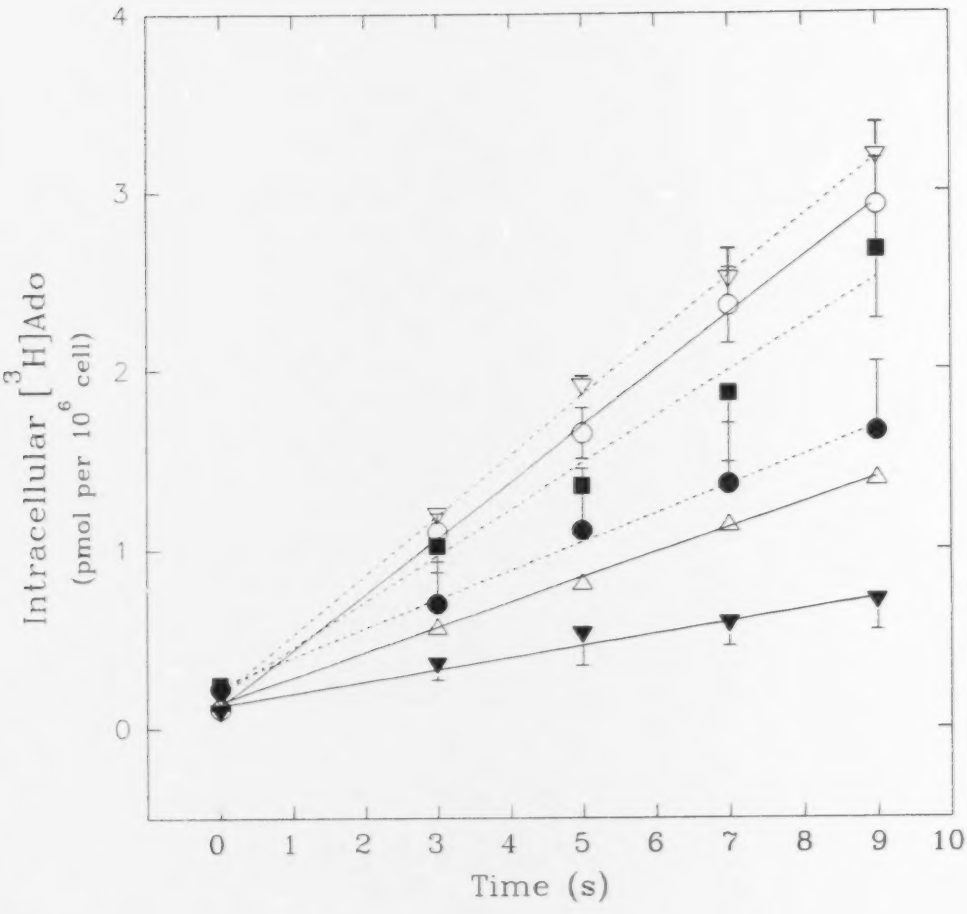

FIG. 13 (C). Concentration-dependence of STA effect on the influx of 1 μM [^{3}H]Ado in CEM cells. Cells were treated for 16 h with 0.1% (v/v) DMSO (vehicle) (O), 1 (∇), 10 (■), 100 (●), 200 (Δ) or 400 nM (▼) STA. Subsequently, cells were washed and resuspended in RPMIt. Uptake of [^{3}H]Ado was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 10 separate experiments, each performed in triplicate.

		1.0 μM [^{3}H]Ado influx rate (pmol per s per 10^6 cells)
Control (0.1% vehicle)		0.31
[STA] (nM)	1	0.32
	10	0.26
	100	0.15*
	200	0.13*
	400	0.07*

Table 9. Rates of [^{3}H]Ado influx in CEM cells treated for 16 h with 10-400 nM STA. Rates were calculated from progress curves in Fig. 13 (C). *Significantly different from control values (P<0.05).

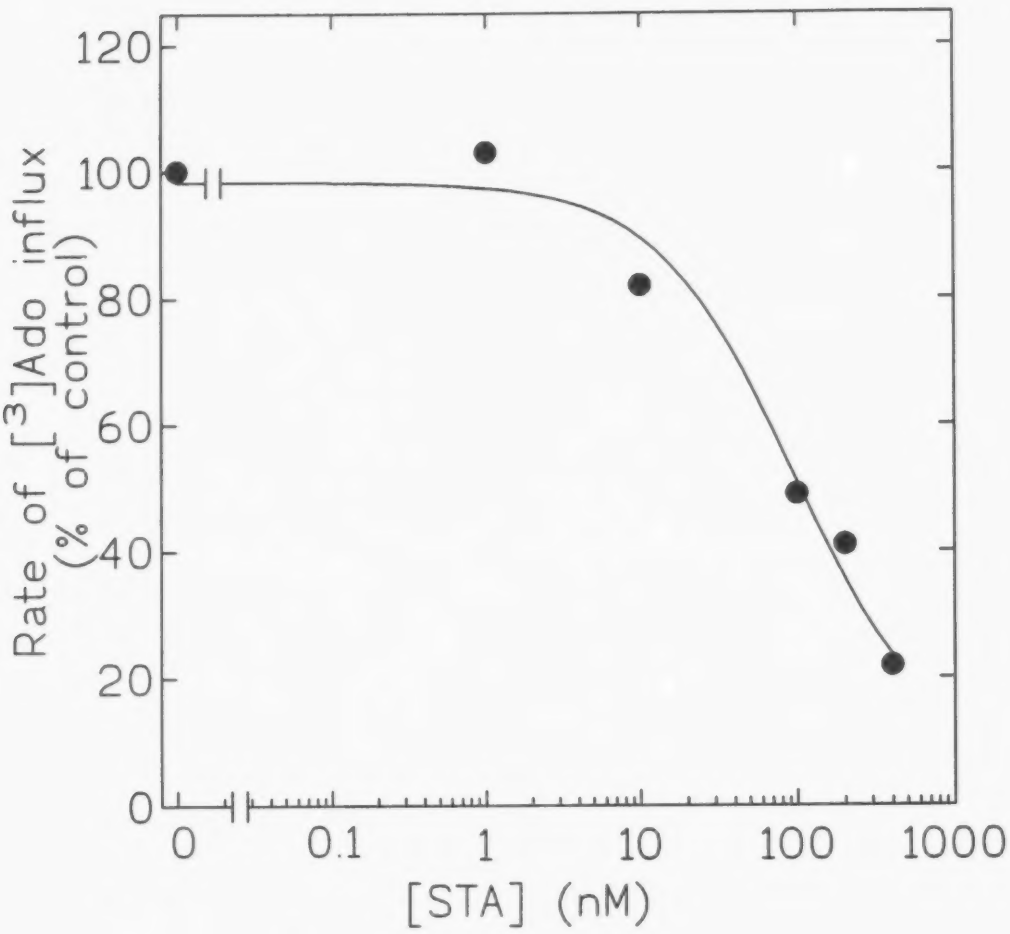

FIG. 13 (D). The effect of increasing concentration of STA on the rate of influx of 1 μM [^{3}H]Ado in CEM cells. Rates of influx of [^{3}H]Ado obtained as slopes of progress curves in Fig. 13 (C), are expressed as percent of rate in control (vehicle-treated) cells, and plotted against concentration of STA. The inward flux in control cells was 0.31 pmol per s per 10^6 cells.

3.3.7. Effect of PMA and STA on V_{max} and K_m of ado influx

In studies so far described on the effects of PMA or STA treatment of cells, on the rate of Ado influx, a single concentration of Ado (1.0 μM) was measured. The scope of the study was therefore broadened to assess the effects of treatment with PMA or STA on the kinetic constants for Ado influx, K_m and V_{max}. We were particularly interested in determining whether the effects of PMA- and STA-treatment on the V_{max} for Ado influx were similar to their effects on B_{max} for NBMPR binding.

Rates of Ado influx were measured at graded [^{3}H]Ado concentrations from 1 to 500 μM. Fluxes were measured in vehicle-treated cells (control), and 16-h, 100 nM PMA- or STA-treated cells. Inward fluxes were calculated as the slopes of lines fitted to data by linear regression, to data that were initial rates of [^{3}H]Ado uptake. Direct plots of rates of Ado uptake against concentration of Ado in control cells, PMA- or STA-treated cells are represented in Fig. 14A. The concentration-dependence of Ado influx was hyperbolic, suggesting that the transport mechanism conformed to Michaelis-Menten kinetics. Incubation of cells for 16 h with 100 nM each of STA or PMA, resulted in a decline in the V_{max}, with incubation of cells in the presence of PMA causing a greater reduction than that caused by STA.

The data of Fig. 14A were transformed and replotted in the form of a Hanes plot (Fig. 14B). Values of V_{max} (determined as the inverse of the slope) were 15.2 pmol/10^6 cells/s, 11.9 pmol/10^6 cells/s, and 8.7 pmol/10^6 cells/s for DMSO, vehicle-treated cells (control), 16-h, 100 nM STA-treated, and 16-h, 100 nM PMA-treated CEM cells respectively. K_m values determined as the x-intercept, were 88.2, 97.3, 91.5 μM respectively for control cells, STA- and PMA-treated cells. These observation are consistent with the findings in studies (above) of equilibrium binding of NBMPR in drug-treated cells, in which preincubation of cells with STA or PMA decreased B_{max} for NBMPR binding without significantly affecting the equilibrium dissociation constant, K_D.

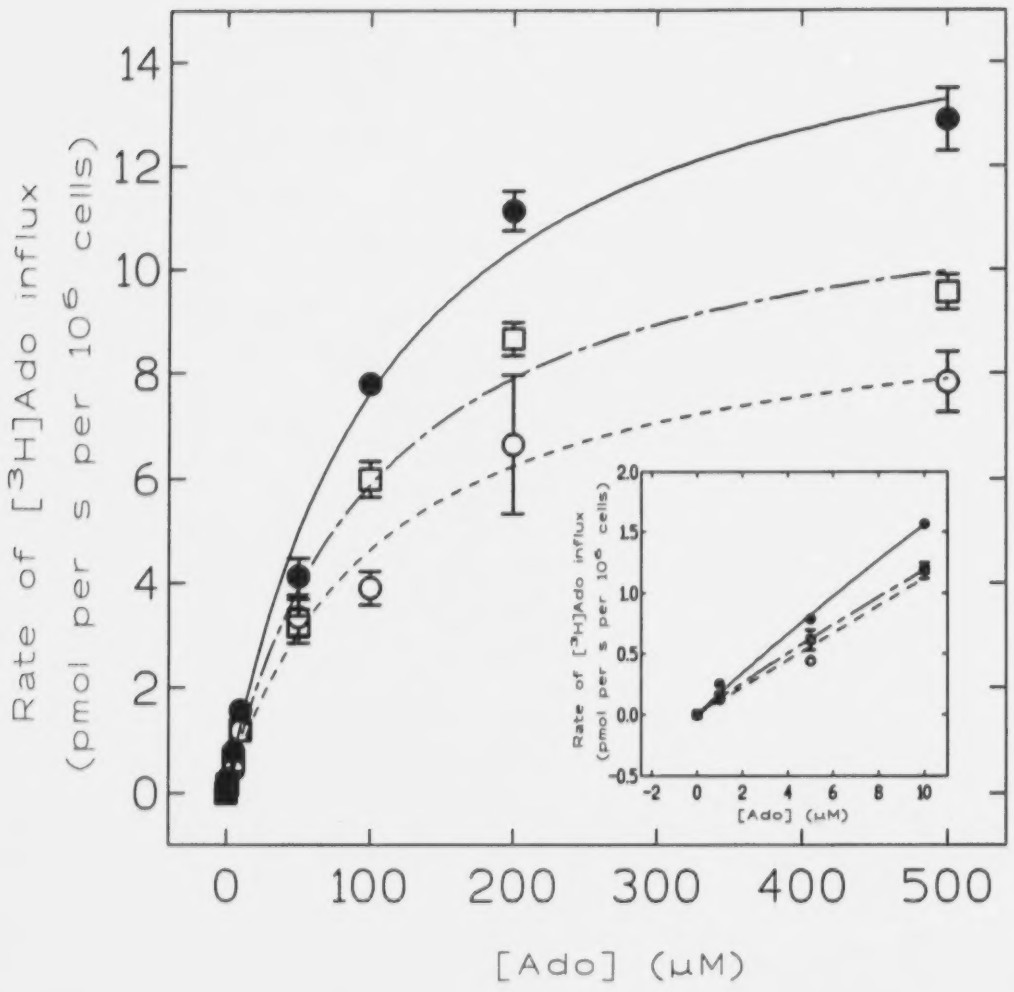

FIG. 14 (A). Effect of treatment of cells with PMA or STA on the kinetic constants of [^{3}H]Ado influx in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM PMA (O) or STA (□), for 16 h. Subsequently, cells were washed and resuspended in RPMIt for measurements of uptake of [^{3}H]Ado. For each concentration of Ado of 1 to 500 μM, fluxes were obtained as the slopes of progress curves that describe [^{3}H]Ado uptake from 3 to 7 s, as described in "Materials and Methods". Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate. Inset is the expanded form of the plot of rate of [^{3}H]Ado influx against [Ado] for concentrations from 0 to 10 μM.

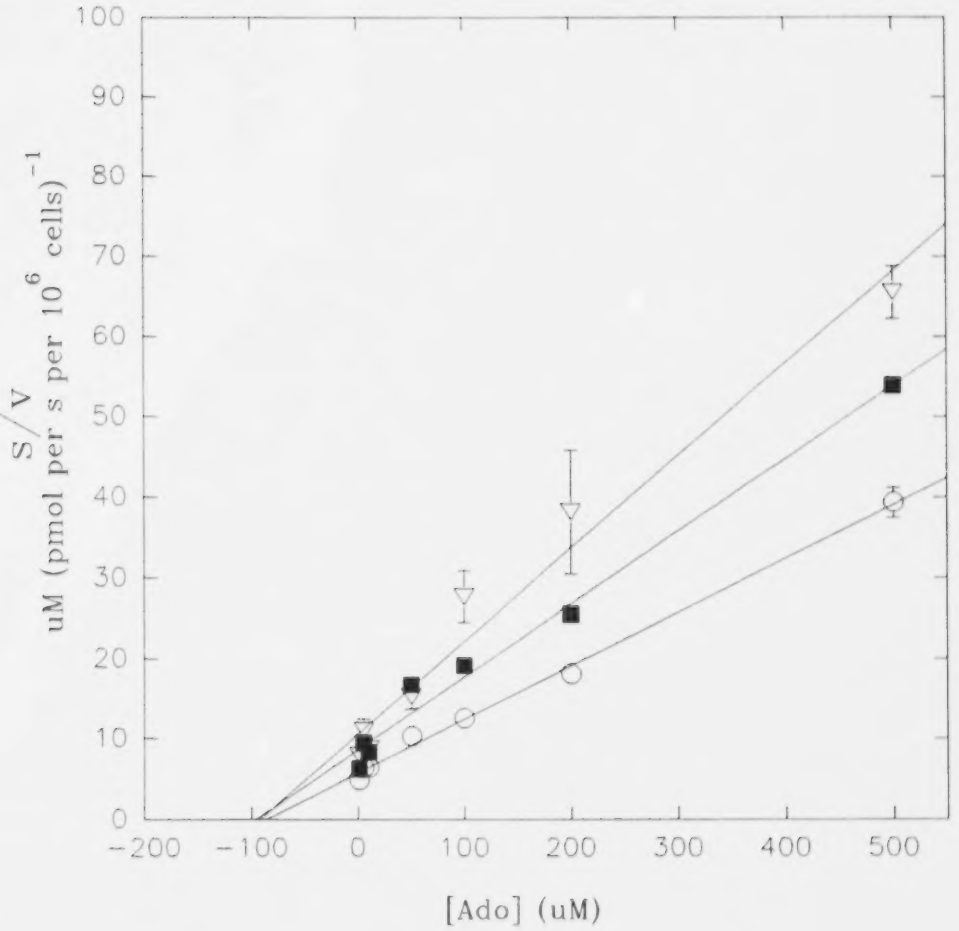

FIG. 14 (B). Hanes plot of the effect of treatment of CEM cells with PMA or STA on V_{max} and K_m of [^{3}H]Ado influx. Data from Fig. 14 (A), which represent rates of [^{3}H]Ado influx in cells treated for 16 h with 0.1% (v/v) DMSO (vehicle) (O), 100 nM PMA (∇) or STA (■), are represented as a Hanes plot. The V_{max} and K_m values are determined from the inverse of slopes of the regression lines and the x-intercept respectively.

3.3.8. Are the effects of treatment of cells with PMA and STA on equilibrium binding of NBMPR and rate of Ado influx in drug-treated CEM cells reversible following subculture?

Our observations showed that prolonged treatment of cells with PMA or STA reduced B_{max} for NBMPR binding, as well as a decline in rate of Ado influx. With this in mind we decided to study both PMA- and STA-treated cells over a period of several days to determine whether the changes in nucleoside transport activity that followed 16-h treatments of cells with these agents were reversible. In this study, therefore, cells were treated with 0.1% (v/v) DMSO (vehicle), or with 100 nM of PMA or STA for 16 h. After treatments, cells were washed, and measurement of influx of 100 μM [^{3}H]Ado and equilibrium binding of [^{3}H]NBMPR were carried out on portion of the cell population while the remaining cells were resuspended in drug-free medium. After 24 h of subculture, measurements of [^{3}H]Ado influx and equilibrium binding of [^{3}H]NBMPR were repeated and the remaining cells again subcultured in drug-free medium for further 24 h. This pattern was repeated until after 72 or 96 h.

Results in Table 10 indicate, as seen previously, that following 16-hr treatment of cells with 100 nM PMA, both the B_{max} for NBMPR binding and rates of influx of 100 μ M Ado were decreased: B_{max} decreased to 33% of the control value, while rate of influx of 100 μM Ado was reduced to 36% of control. In these and subsequent experiments, 100 μM [^{3}H]Ado was used, a concentration similar to the K_m for Ado influx, to provide a more sensitive measure of the loss of transporter activity. Washout of PMA after 16-h treatment, and subsequent subculture of cells in drug-free medium did not reverse the PMA-induced decline in B_{max} for NBMPR binding or in rate of Ado influx, which remained at about 30% of the control value (Table 10). The K_D for NBMPR binding was not significantly altered.

	[^{3}H]NBMPR BINDING		
	B_{max} (molecules per cell x 10^{-4})	K_D (nM)	100 μM Ado influx rate (pmol per s per 10^6 cells)
Control (0.1% vehicle)	17.6 ± 0.9	0.41	13.5 ± 0.6
100 nM PMA, 16 h	5.8 ± 1.1*	0.43	4.8 ± 1.2*
after washout:			
24 h	5.9 ± 1.6*	0.39	3.5 ± 2.1*
48 h	4.6 ± 1.3*	0.33	3.5 ± 1.9*
72 h	5.2 ± 0.4*	0.34	3.3 ± 0.2*
96 h	x		3.6 ± 0.4*
100 nM STA, 16 h	6.4 ± 0.9*	0.42	5.0 ± 0.8*
after washout:			
24 h	6.0 ± 0.5*	0.38	4.3 ± 1.0*
48 h	5.4 ± 0.5*	0.39	2.8 ± 1.2*
72 h	5.3 ± 0.7*	0.43	4.5 ± 0.9*
96 h	x		3.5 ± 0.7*

Table 10. Levels of B_{max} for [^{3}H]NBMPR binding and rates of [^{3}H]Ado influx in PMA- or STA-treated CEM cells. Cells were treated with 100 nM of PMA or STA for 16 h. Subsequently, cells were washed and portions of the suspension were used for measurements of equilibrium binding of NBMPR as well as Ado influx rates. The remaining cells were resuspended in fresh culture medium at 1 x 10^5/ml, and subcultured over 3-4 days. On each day up to day 4, portions of the cell culture were harvested for measurements of Ado influx rate and equilibrium binding of NBMPR. Values are the means ± S.E.M. of two separate experiments performed in triplicates. *Significantly different from control values (P<0.05). x: not determined.

A similar trend was observed in STA-treated cells after treatment and following STA washout, resuspension and subculture in drug-free medium. After a 16 hr incubation of cells with 100 nM STA, B_{max} for NBMPR binding was reduced to 36% of control while influx rate of 100 μM Ado was reduced to 37% of control. Again, K_D was not significantly altered (Table 10). After washout of STA, drug-induced decline in B_{max} for NBMPR binding and rates of Ado influx did not return to control values, but remained at about 30% of control values. These results shows that the effects of PMA and STA on nucleoside transporters persisted for at least 3 days after drug removal, suggesting that a long-lasting change in binding sites numbers as well as transporter activity had occurred.

3.3.9. Effect of treatment of CEM cells with other agents on rate of ado influx

The involvement of PKC in the STA-mediated decrease in Ado influx rate was addressed, since STA is not specific for inhibition of PKC (74). Ado influx rates were measured in cells pretreated for 16 h with HA1004, a PKA and cGMP-dependent protein kinase inhibitor, genistein, a tyrosine kinase inhibitor, and H7, another non-specific PKC inhibitor. Results in Fig. 15A show that the rate of influx of 100 μM Ado in cells pretreated with HA1004 or genistein did not differ from the rate obtained in vehicle-treated cells (control) (Fig. 15A). Rates of Ado influx in control cells and in cells treated with HA1004 or genistein were 13.5, 13.8 and 14.4 pmol per s per 10^6 cells respectively. In contrast, rates were significantly reduced in cells treated with STA or H7, from the control rate of 13.5 to 5.0 and 3.8 pmol per s per 10^6 cells respectively (Figs. 15A and B). These results indicate that STA or H7 did not affect Ado influx through interaction with PKA, cGMP-dependent protein kinase, or tyrosine kinase. Also shown in Figs. 15A and B is the observation that in cells pretreated with 4α-PDD, an inactive phorbol ester, rate of influx of 100 μM Ado did not change significantly, in contrast with cells preincubated for 16 h with 100 nM each of PMA or PDBu, in which rates of influx of 100 μM Ado decreased from control (13.5 pmol per s per 10^6 cells) rate to 4.8 and 3.5 pmol per s per

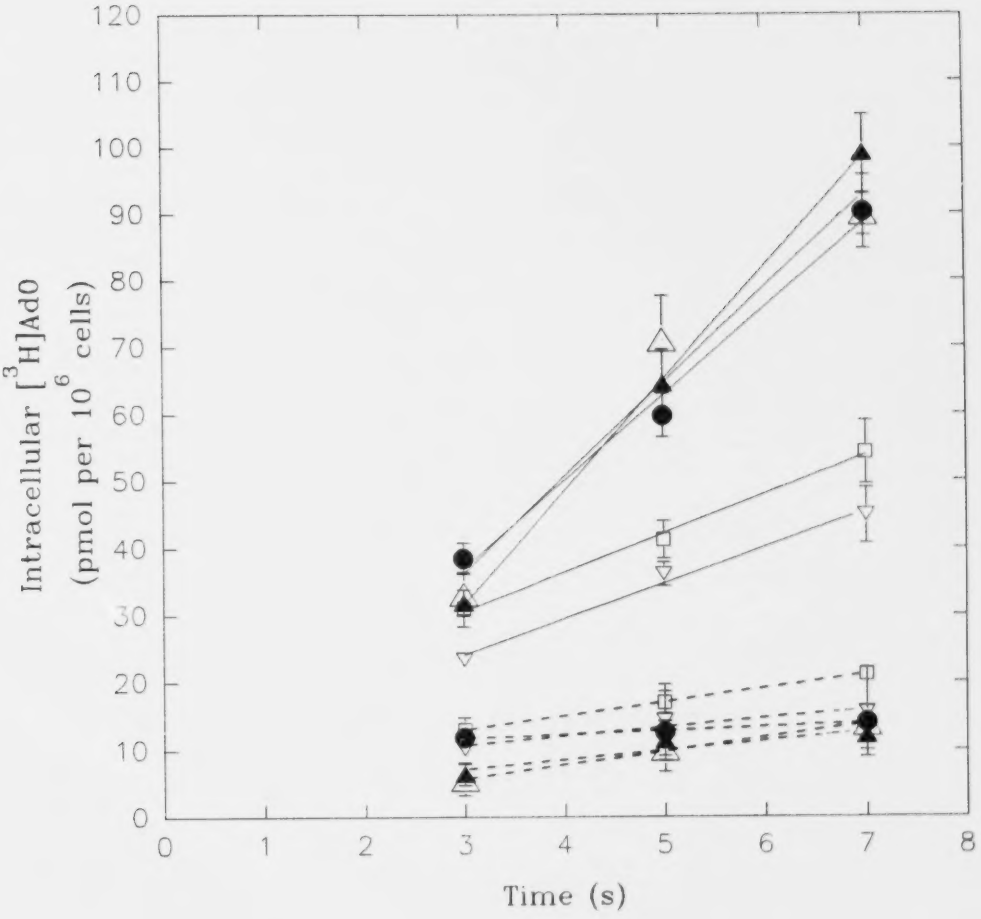

FIG. 15 (A). Time-course of uptake of 100 μM [^{3}H]Ado in CEM cells treated with various agents. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), 40 μM HA1004 (▲), 50 μM genistein (Δ), 100 nM PMA (∇) or 100 nM STA (□) for 16 h. Subsequently, cells were washed and resuspended in RPMIt. Uptake of [^{3}H]Ado (solid lines) was measured as described in "Materials and Methods". Measurements of uptake of [^{3}H]Ado in the presence of 5 μM NBMPR (dashed lines) were also carried out in cells from each group. Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate.

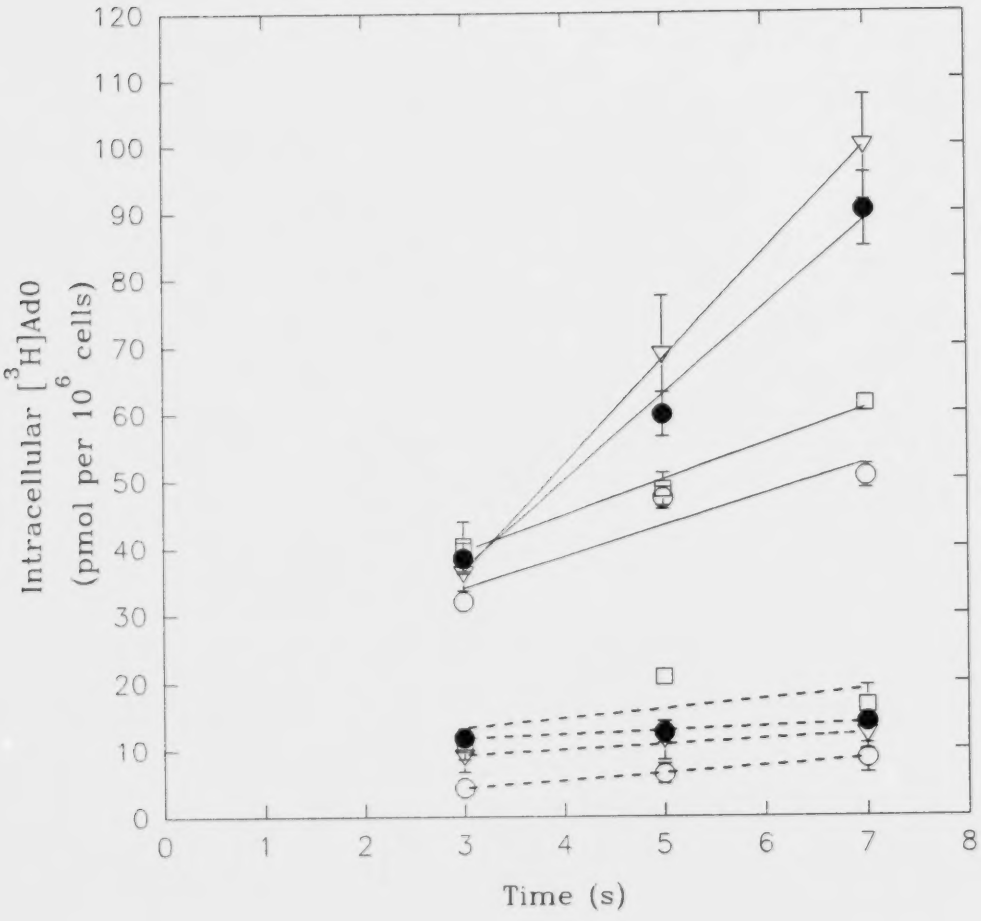

FIG. 15 (B). Time-course of uptake of 100 μM [^{3}H]Ado in CEM cells treated with various agents. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), 100 nM 4α-PDD (▽), 100 nM PDBu (○) or 40 μM H7 (□), for 16 h. Cells were subsequently washed and resuspended in RPMIt. Uptake of [^{3}H]Ado in the presence (dashed lines) or in the absence (solid lines) of 5 μM NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate.

10^6 cells respectively. Time course of [^{3}H] Ado (100μM) uptake into control cells, and into 16-h drug-treated cells in presence of 5 μM NBMPR are also represented in Fig. 15A and B. The reduction in rates of Ado influx to near zero in the presence of NBMPR is in agreement with literature reports that only NBMPR-sensitive facilitated diffusion nucleoside transporters are expressed in CEM cells (102). The data in Fig. 15A and B show that pretreatment of cells with PMA, STA, or other agents did not induce NBMPR-insensitive transporter activity in CEM cells.

In summary, it is clear that prolonged treatment of CEM cells with PMA or with STA, resulted in substantial decline in B_{max} for NBMPR binding and in Ado influx rates. Since prolonged incubation was necessary for PMA effect to be observed, it is not likely that PMA-induced PKC activation is the underlying basis for the changes observed, because PMA-induced PKC activation may be achieved in minutes (40, 90, 91). Besides, prolonged treatment of cells with phorbol ester is known to result in PKC down-regulation (6, 29, 44). Additional support for the idea that PKC down-regulation underlies the observed changes in nucleoside transporter activity is the fact that treatment of cells with STA achieved similar results to those observed following prolonged treatment with PMA, since the effects of down-regulation of PKC and PKC inhibition should be similar.

3.3.10. Effect of short treatment of cells with PMA on equilibrium binding of NBMPR and rate of ado influx

The reduction in the NBMPR binding site numbers and nucleoside transporter activity in CEM cells had occurred as a result of prolonged exposure of cells to PMA, and thus suggested that down-regulation of PKC was the basis for the change. In another approach to study the effect of PKC activity changes on nucleoside transporter activity, CEM cells were exposed to PMA for short times (minutes) with the aim of activating PKC, and subsequently, the number of high affinity NBMPR binding sites and transporter activity were measured. In cells treated with 100 nM PMA for 10 min, the B_{max} for

NBMPR binding was not significantly different from that obtained in control (vehicle-treated) cells (Fig. 16A). Values of B_{max} in control (vehicle-) cells and in cells treated with 100 nM PMA for 10 min are 17.4 x 10^4 and 17.0 x 10^4 molecules/cell respectively; K_D value was unchanged at 0.39 nM.

The uptake of [^{3}H]Ado measured in vehicle-treated cells and in cells treated with 100 nM PMA, for 5 or 10 min were not significantly different. Rates of Ado influx in control cells were 12.7 pmol per s per 10^6 cells, while rates measured in cells treated for 5 or 10 min, with 100 nM PMA were 12.9 and 13.1 pmol per s per 10^6 cells respectively. Also shown is the uptake of [^{3}H]Ado control cells and PMA-treated cells in presence of 5 μM NBMPR which nearly completely blocked the influx of Ado, suggesting that the transporter was still NBMPR-sensitive and also that no inhibitor-insensitive transporter had been induced during treatment with PMA. These results also indicate that a short time of incubation of cells with PMA did not result in significant changes in NT activity.

3.3.11. Effect of PMA on NT activity following treatment of cells with STA for 16 h

Another approach to investigate the role of PKC in NT activity was based on the idea that cells treated for 16 h with STA would lose PKC activity, and that such cells, if exposed to PMA for a short period of time, might regain normal levels of PKC activity, and might show a reversal of the STA-mediated decrease in transporter activity. The rate of 100 μM [^{3}H]Ado influx following treatment of cells with 100 nM STA for 16 h, and subsequently with 100 nM PMA for 10 min, was 3.1 which was not significantly different from the rate measured in 16-h, 100 nM STA-treated cells, which were subsequently treated with 0.1% (v/v) DMSO (vehicle) of 1.8 pmol per s per 10^6 (Fig. 17). Both values of the rates were significantly different compared with that obtained in control (vehicle-treated) cells, of 13.8 pmol per s per 10^6 cells. The rates of Ado influx measured in the presence of 5 μM NBMPR, showed that after treatment of cells with PMA or STA, the transporter was still sensitive to inhibition by NBMPR.

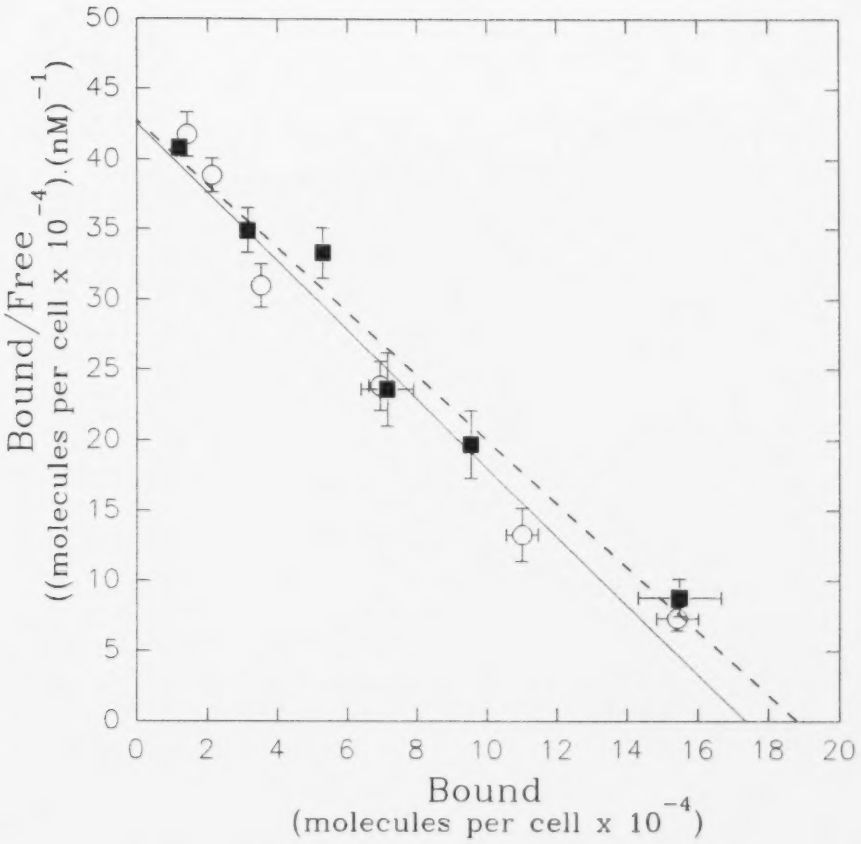

FIG. 16 (A). Scatchard analysis of the effect of short treatment duration with PMA on equilibrium binding of [^{3}H]NBMPR in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (O) or 100 nM PMA (■) for 10 min. Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Results are from at least 3 separate experiments, each performed in triplicate.

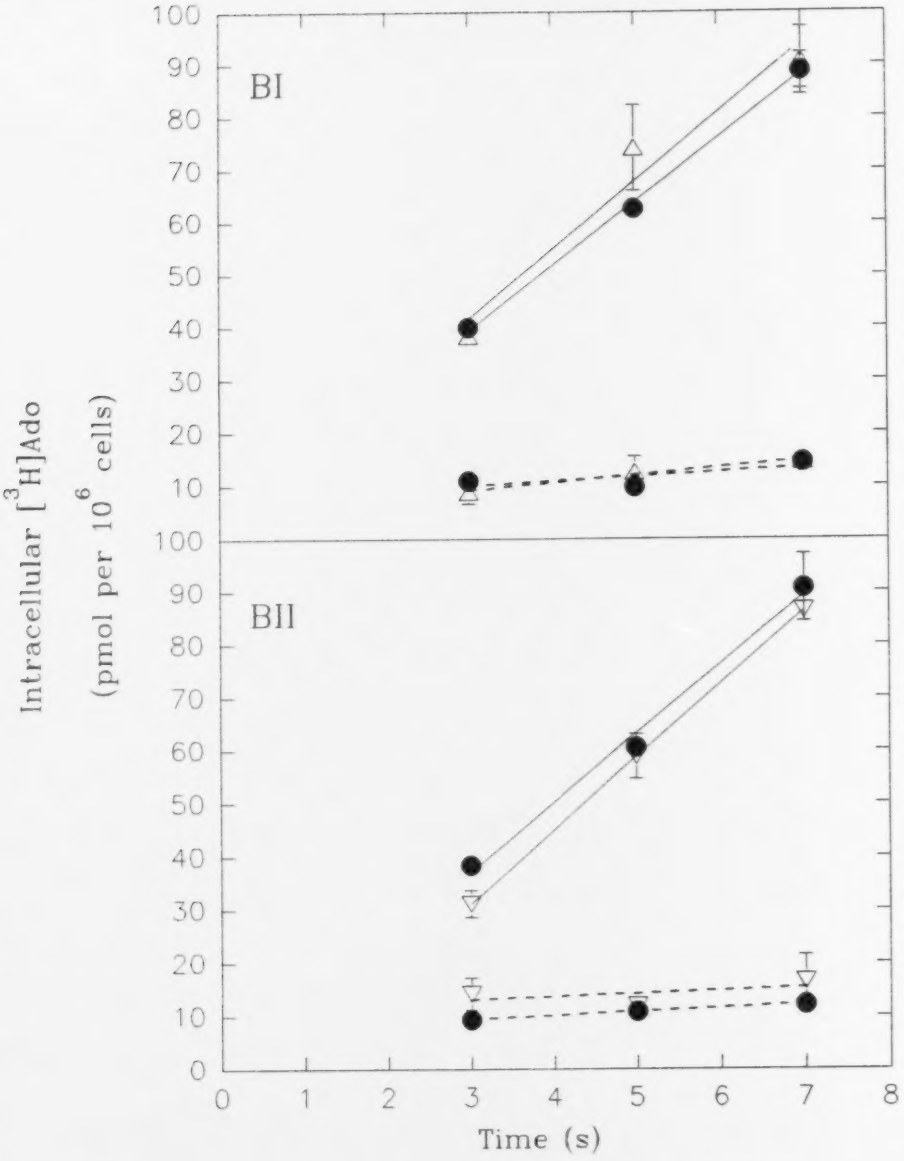

FIG. 16 (B). Effect of short treatment duration with PMA on the influx of 100 μM [^{3}H]Ado influx in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM PMA for 5 min (panel B[I]) (Δ), or 10 min (panel B[II]) (∇) prior to measurements of uptake of [^{3}H]Ado in the presence (dashed lines) or in the absence (solid lines) of 5 μM NBMPR. Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate.

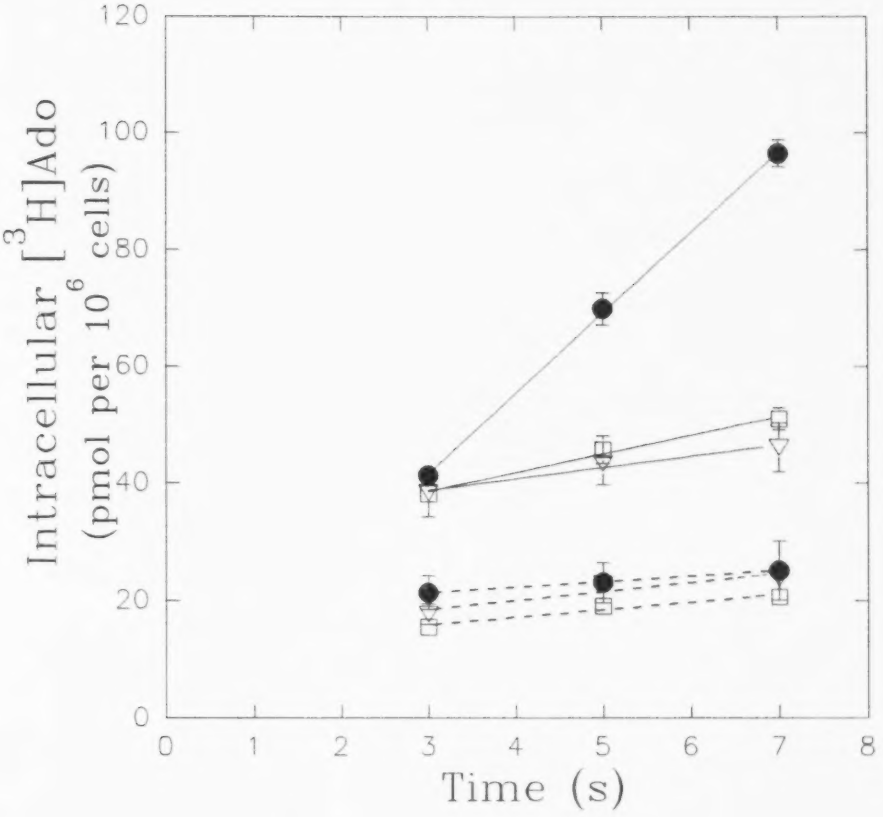

FIG. 17. Effect of PMA on the time-course of uptake of 100 μM[^{3}H]Ado in STA-treated CEM cells. Cells were treated with 100 nM STA for 16 h prior to treatment with 0.1% (v/v) DMSO (vehicle) (□) or 100 nM PMA for 10 min (▽). For another control, cells were treated with 0.1% (v/v) DMSO (vehicle) for 16 h prior to a second treatment with vehicle for 10 min (●). Following these treatments, cells were washed, resuspended in fresh growth media and allowed to rest at 37°C for 2 h. Subsequently, cells were washed and resuspended in RPMIt and measurements of uptake of 100 μM [^{3}H]Ado in the presence (dashed lines) and in the absence (solid lines) of 5 μM NBMPR were carried out as described in "Materials and Methods". Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

In studies so far, it was observed that modulation of PKC resulted in reduction in B_{max} of NBMPR binding and in Ado influx rate. These changes were evident only when PKC was inhibited or down-regulated. This means that for the regulation of the number high affinity NBMPR binding sites and nucleoside transporter activity, PKC activity is required. Furthermore, conditions that were capable of activating PKC did not result in increased B_{max} of NBMPR binding or Ado influx rate. Therefore, NT activity and binding sites numbers for NBMPR on transporter protein may be at the maximum in CEM cells when basal PKC activity is unperturbed.

3.4. EFFECT OF OKADAIC ACID (OKA) AND CALYCULIN A (CLA) ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND INWARD FLUXES OF ADO IN CEM CELLS

The results so far indicated that a decrease in PKC activity was the cause for the reduction in number of high affinity NBMPR binding sites and nucleoside transporter activity. Thus it was reasoned that PKC-mediated phosphorylation steps may be required in the mechanism of maintaining the highest level of nucleoside transporter. Since cycles of phosphorylation and dephosphorylation are known to regulate the activity of some cellular enzymes, it was thought that inhibition of the dephosphorylation step may provide some further insights on the dependence of NT activity on PKC-mediated phosphorylation. Therefore experiments were conducted using protein phosphatase inhibitors that would block dephosphorylation steps. Effects of treatment of cells with protein phosphatase inhibitors, okadaic acid (OKA) and calyculin A (CLA), on the equilibrium binding of NBMPR and on the rate of Ado influx were measured. Results in Fig. 18A show that treatment of cells with OKA alone did not change B_{max} of NBMPR binding. However, in cells treated for 16 h with 100 nM STA, B_{max} was reduced to 7.4×10^4 molecules/cell; in cells treated with both STA and OKA, B_{max} was 12.9×10^4 molecules/cell, which was

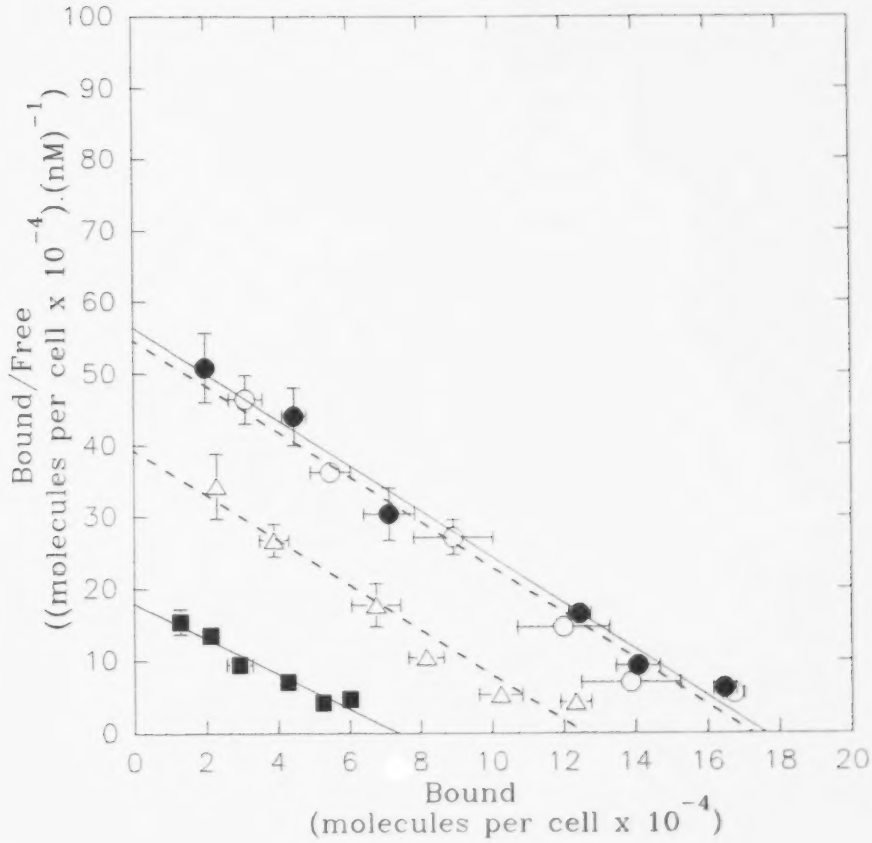

FIG. 18 (A). Scatchard analysis of the effect of treatment of CEM cells with OKA on the equilibrium binding of [^{3}H]NBMPR. Cells were treated for 16 h with 0.1 % (v/v) DMSO (vehicle) (●, solid line), 10 nM OKA (O, dashed line), 10 nM OKA plus 100 nM STA (Δ, dashed line) or 100 nM STA (■). Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of at least 5 separate experiments, each performed in triplicate.

cells preincubated for 16 h with both STA (100 nM) and OKA (10 nM), the decrease in B_{max} observed in STA-treated cells was partially blocked by the presence of OKA. In near the control value of 17.4 x 10^4 molecules/cell (Fig. 18A).

The observation that OKA alone did not have any effect on B_{max} agrees with the concept that NT may be maximally active at basal PKC activity, and that any further enhancement in the PKC-mediated phosphorylation, or in the level of putative phosphorylated protein (either transporter protein or some other protein), would not result in elevation in NT activity. The latter case would be achieved by inhibition of protein phosphatases. Furthermore, the finding that OKA partially blocked STA-mediated decline in B_{max} of NBMPR binding sites when present in cells in combination with STA, shows that blockade of protein phosphatase-mediated dephosphorylation might keep a putative phosphoprotein at a level that is adequate for maintenance of NBMPR binding sites and NT activity, even though PKC may be inhibited by STA. This also confirms that protein phosphorylation and dephosphorylation are necessary steps in the regulation of NT activity. Similar results were obtained when cells were incubated simultaneously with 100 nM STA and 10 nM CLA for 6 h. In cells treated for 6 h with 10 nM CLA, B_{max} of NBMPR binding was not significantly different from B_{max} value obtained in 0.1% (v/v) ethanol, vehicle-treated cells (16.4 x 10^4 molecules/cell for cells treated with CLA, and 17.4 x 10^4 molecules/cell in control cells) (Fig. 18B). However, when cells were treated for 6 h with both CLA and STA (in the same solution), STA-mediated decline in B_{max} of NBMPR binding was not seen. The value of B_{max} in cells treated for 6 h with STA was 10.8 x 10^4 molecules/cell, while B_{max} in cells simultaneously treated with CLA and STA was 15.3 x 10^4 molecules/cell (Fig. 18B). The difference in the abilities of OKA and CLA to prevent the STA-induced decline in B_{max} may be due to the fact that CLA is equally potent in inhibiting protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), while OKA is more potent against PP2A than PP1 (180-182). The results of the study

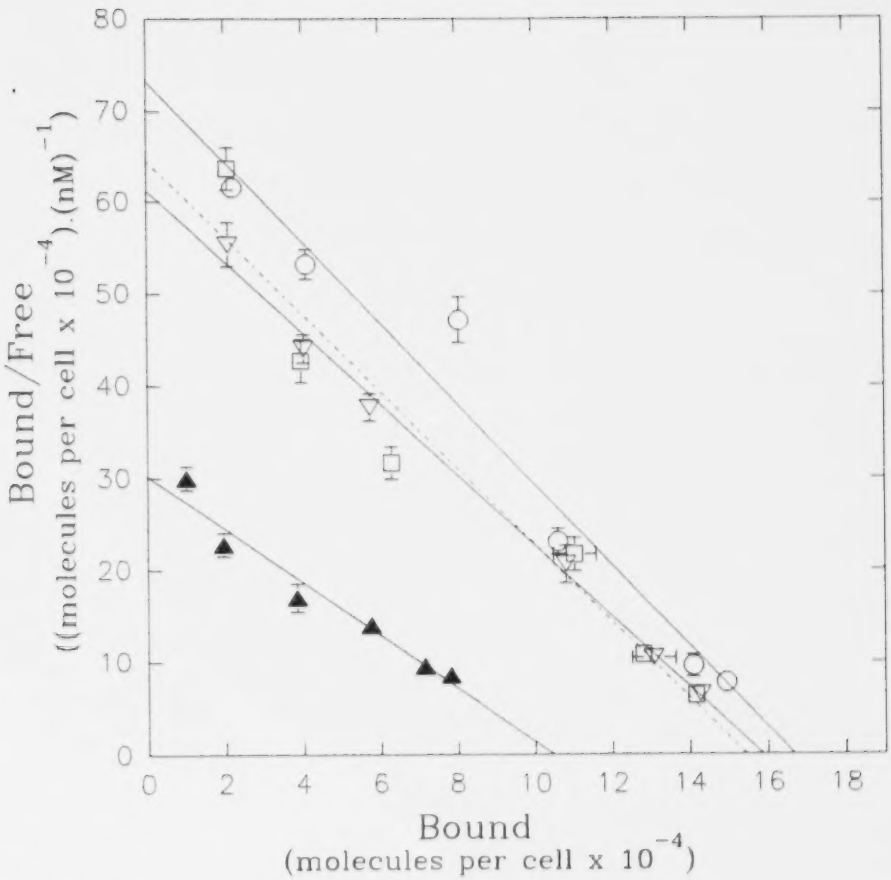

FIG. 18 (B). Scatchard analysis of the effect of treatment of CEM cells with CLA on the equilibrium binding of [^{3}H]NBMPR. Cells were treated for 6 h with 0.1% (v/v) ethanol (vehicle) (O), 10 nM CLA (□), 10 nM CLA plus 100 nM STA (▽) or 100 nM STA (▲). Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [^{3}H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

involving CLA agree with the study with OKA, and further support the concept that phosphorylation and dephosphorylation are essential steps in the regulation of the cellular abundance of NBMPR binding sites and NT activity.

The effects of the two phosphatase inhibitors on rate of Ado influx in STA-treated cells were also examined. OKA alone did not have significant effect on rate of influx of Ado. Contrary to expectations, however, incubation of cells with both OKA and STA together did not block the STA-induced decrease in rate of Ado influx (Fig. 18C). Rates of influx of Ado in vehicle-treated and in OKA-treated cells, were 12.4 and 12.5 pmol per s per 10^6 cells respectively. Rates of Ado influx in STA-treated cells, and in cells treated simultaneously with STA and OKA, were 4.18 and 4.17 pmol per s per 10^6 cells respectively (Fig. 18C). Also shown in Fig 18C is Ado uptake in vehicle-treated cells, and in drug-treated cells, measured in the presence of 5 μM NBMPR, which blocked uptake of Ado, indicating that the transporter remained NBMPR-sensitive.

Results in Fig. 18D also show that CLA alone did not have any effect on rate of Ado influx, however, CLA completely reversed STA-mediated decrease in Ado influx rate. The rate of Ado influx in cells exposed for 6 h to 100 nM STA was 6.3 pmol per s per 10^6 cells, and this was increased to 11.8 pmol per s per 10^6 cells in cells simultaneously treated with 10 nM CLA and 100 nM STA. Rate of Ado influx in control cells (0.1% (v/v) ethanol, vehicle-treated cells) was 13.9 pmol per s per 10^6 cells, while the rate in cells treated for 6 h with 10 nM CLA was 13.7 pmol per s per 10^6 cells. As was stated before, the differences in the effects of OKA and CLA on rate of Ado influx may be due to the fact that CLA is equally potent against PP1 and PP2A while OKA is more potent against PP2A than PP1 (180-182). Fluxes of Ado measured in vehicle-treated and in CLA-treated cells, in the presence of 5 μM NBMPR showed that the transporter remained NBMPR-sensitive in control cells and in cells treated with CLA and that no inhibitor-insensitive transporters had been induced (Fig. 18D). Rates measured in the presence of NBMPR were minimal.

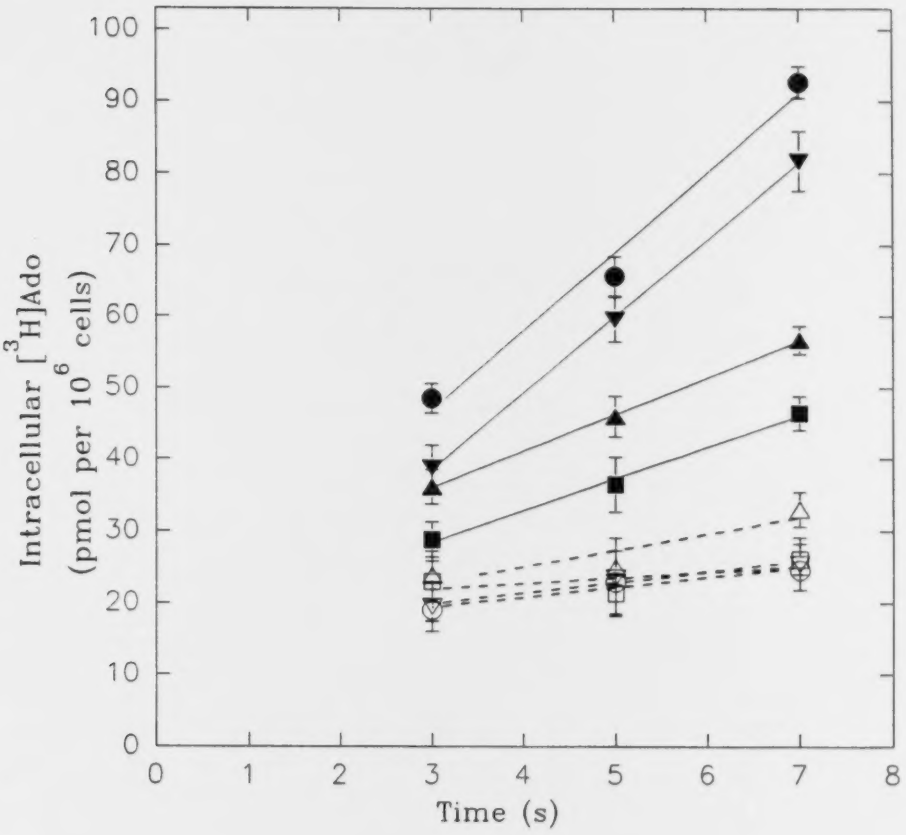

FIG. 18 (C). Effect of treatment of cells with OKA on time-course of 100 μM [^{3}H]Ado uptake in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●, O), 10 nM OKA (▼,▽), 10 nM OKA plus 100 nM STA (■,□) or 100 nM STA (▲, △) for 16 h. Subsequently, cells were washed and resuspended in RPMIt and uptake of [^{3}H]Ado was measured as described in "Materials and Methods". Closed and open symbols represent uptake of [^{3}H]Ado measured in the absence and in the presence of 5 μM NBMPR respectively. Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

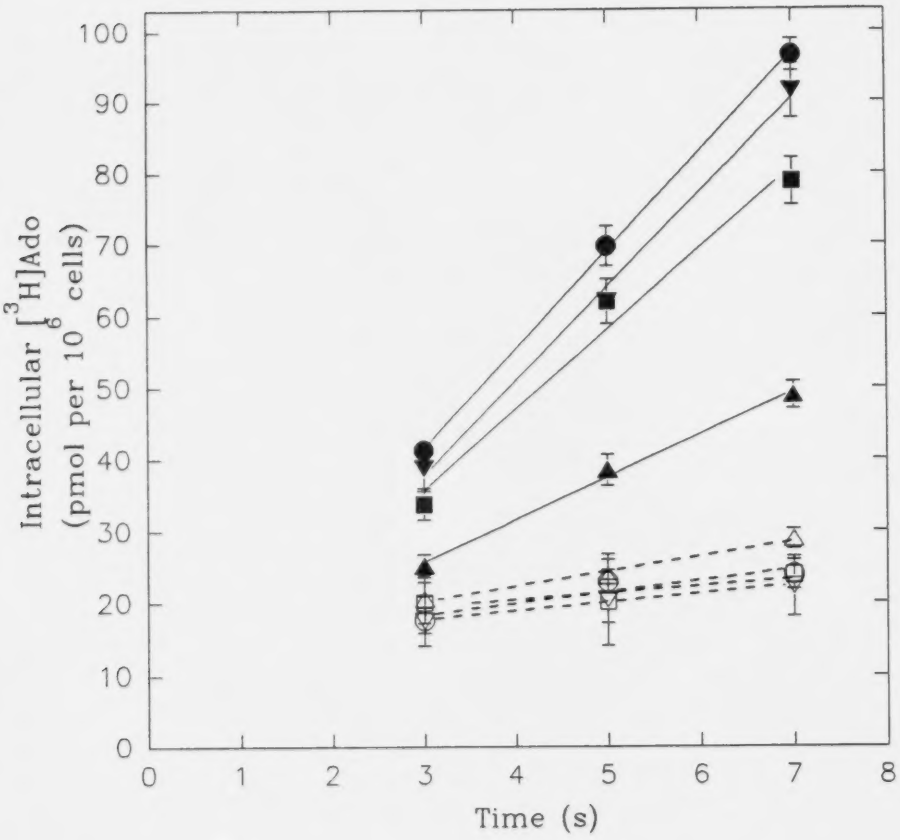

FIG. 18 (D). Effect of treatment of cells with CLA on time-course of 100 μM [^{3}H]Ado uptake in CEM cells. Cells were treated with 0.1% (v/v) DMSO (vehicle) (●, O), 10 nM CLA (▼,▽), 10 nM CLA plus 100 nM STA (■,□) or 100 nM STA (▲,△) for 6 h. Subsequently, cells were washed and resuspended in RPMIt and uptake of [^{3}H]Ado was measured as described in "Materials and Methods". Closed and open symbols represent uptake of [^{3}H]Ado measured in the absence and in the presence of 5 μM NBMPR respectively. Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

4. DISCUSSION

4.1. NON-PKC SPECIFIC EFFECTS OF STAUROSPORINE ON INOSITOL PHOSPHATE SIGNAL TRANSDUCTION IN HUMAN PLATELETS

4.1.1. STA STIMULATES HYDROLYSIS OF PHOSPHATIDYL INOSITOL 4,5-BISPHOSPHATE IN HUMAN PLATELETS

The effects of STA, a non-specific PKC inhibitor, on the inositol phosphate signal transduction was studied to assess the non-PKC-specific effects of this inhibitor. Results of the studies described herein, which have already been published (207), indicate that micromolar concentrations of STA cause hydrolysis of polyphosphoinositide in intact human platelets. STA induced both time- and concentration-dependent formation of $InsP_3$ (Fig. 2) (207). At STA concentrations of 1-2 μM, production of $InsP_3$ plateaued after 15 s, in contrast to levels produced in response to the physiological agonist, thrombin (Fig. 2), which is known to peak at 10 s and rapidly decline back to basal levels again (89).

Physiologically, hydrolysis of PIP_2 is achieved by the action of phosphoinositide-specific phosphoinositidase, PLC, in response to agonist (4). This produces Ins $(1,4,5)P_3$ and DAG (4, 61), two second messengers that mobilize intracellular Ca^{2+} from storage sites (46, 62) and activate PKC, respectively (45, 62). In this study, STA, a PKC inhibitor, was found to act like an agonist, stimulating the hydrolysis of PIP_2 with the formation of $InsP_3$ (Fig. 2) (207). STA-induced formation of $InsP_3$ was sustained (Fig. 2). This might be due to the simultaneous inhibition of PKC-induced degradation of $InsP_3$. PKC is known to regulate two key enzymes that metabolize $Ins(1,4,5)P_3$, $InsP_3$ kinase and $InsP_3$ 5-phosphatase (89), which convert $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ and $Ins(1,4)P_2$ respectively. The idea that STA-induced $InsP_3$ formation is via activation of PLC-mediated PIP_2 hydrolysis is further supported by the observation that neomycin

completely blocked STA-induced $InsP_3$ production (Fig. 3) (207). Neomycin is known to inhibit PLC-mediated hydrolysis of PIP_2 by blocking PLC (91). By blockade of PLC, STA-induced activation of PLC-PIP_2 hydrolysis was cirumvented.

The possibility that STA might be mediating an indirect activation via thromboxane A_2 (TXA_2) generation is unlikely. This is because work in the Wong laboratory that assayed for the TXA_2 metabolite, TXB_2, by radioimmunoassay did not detect significant levels of the metabolites (207). STA may directly act on a molecular target anywhere along the phosphoinositide pathway preceeding PLC, to eventually cause activation of PIP_2 hydrolysis, as the results of the study with neomycin (Fig. 3) would also suggest.

The possibility of a G protein being the site of action of STA was investigated using the non-hydrolyzable GTP analog, GTPγS, in permeabilized platelets. The results provide further evidence substantiating the hypothesis that the STA-induced formation of $InsP_3$ occurs via activation of the PLC-PIP_2 pathway. In the study of STA effects on [^{3}H]-inositol labeled saponin-permeabilized human platelets, results show that the PKC inhibitor caused increased formation of $InsP_3$ above basal levels similar to the observation in intact platelets (Fig. 4). Furthermore, in the presence of GTPγS, STA caused an increased formation of $InsP_3$ higher than levels observed when either one of the two agents was used alone (Fig. 4). The observed effect of the two agents albeit simultaneously, did not suggest synergism between the actions of the two agents. This suggests that the two agents may act at the same molecular target along the PLC-PIP_2 signal transduction pathway. In such a situation, the same molecular target would be activated by both agents with the action of each agent contributing to the activation process. GTPγS, a non-hydrolyzable GTP analog, would persistently activate G proteins (186, 187), causing activation of PLC-mediated hydrolysis of PIP_2 in the absence of a physiological agonist.

Based on this observation, it was conceived that STA may be affecting the G protein that couples the agonist receptor to PLC, which is also the same molecular target

for the action of GTPγS. Or perhaps STA may affect some other target, a receptor, which in turn would alter the G protein function to activate PLC activity. However, experiments conducted later (discussed in section 4.1.3.), and direct evidence reported by Kanaho and others (214) indicate that STA directly activates the G protein which would consequently lead to PLC-induced hydrolysis of PIP_2 and $InsP_3$ formation.

Studies carried out to measure levels of $Ins(1,4,5)P_3$ by a $Ins(1,4,5)P_3$ radioligand binding assay, showed that STA caused significant formation of $Ins(1,4,5)P_3$ in intact platelets (Fig. 5). Levels of $Ins(1,4,5)P_3$ induced by STA were, however, lower than levels of $InsP_3$ measured in [^{3}H]myo-inositol labeled platelets by anion exchange chromatography (Figs. 2, 3). One factor that may account for this difference is the fact that $InsP_3$ levels measured by anion exchange chromatography include all isomers of $InsP_3$ while only one isomer, $Ins(1,4,5)P_3$, was measured by the radioligand binding assay. Another reason may be the fact that $InsP_3$ levels measured by anion exchange chromatography may contain some contaminants of inositol mono- and bis-phosphates (184) although washing using the appropriate buffers should remove these contaminants.

Contrary to results observed here, Murphy and others (215) have reported no effect of 1 μM STA on $Ins(1,4,5)P_3$ levels in rabbit platelets. This difference in results may be accounted for by species differences.

4.1.2. EFFECT OF STA ON $[CA^{2+}]_i$ IN HUMAN PLATELETS

A puzzling observation was the fact that STA induced a weak elevation in $[Ca^{2+}]_i$ in platelets (Fig. 1) compared to the levels of $InsP_3$ induced by this agent (Fig. 1). The possibility that STA blocks STA-induced $Ins(1,4,5)P_3$ from mobilizing intracellular Ca^{2+} is ruled out due to the fact that the PKC inhibitor potentiated Ca^{2+} mobilization induced by $Ins(1,4,5)P_3$ formed as a result of thrombin-induced PIP_2 hydrolysis (Fig. 1). Various reasons could be invoked to explain the weak $[Ca^{2+}]_i$ mobilization. First, the levels of

Ca^{2+}-mobilizing second messenger, Ins(1,4,5)P_3, induced by STA are lower than those observed in response to thrombin (Fig. 1). Second, initial increases in $[Ca^{2+}]_i$ triggered by platelet agonists like thrombin are known to be amplified by phospholipase A2 activation and TXA_2 formation (47, 61, 216). This signal leading to amplification may be absent in the STA-mediated responses since it has been found that STA does not activate TXA_2 formation (207). Third, it has recently been reported that agonist-induced mobilization of Ca^{2+} may have a small contribution from Ca^{2+} influx. This observation, made by Geiger and others (217), showed that even after inhibition of thrombin-stimulated Ca^{2+} mobilization through stimulation of PKA or cGMP-dependent kinase, there was still observed a small influx of Ca^{2+} (217). The additional influx of extracellular Ca^{2+} may be absent in STA-mediated $[Ca^{2+}]_i$ mobilization. For these reasons, STA-induced $InsP_3$ formation may not produce a $[Ca^{2+}]$-mobilizing response to the same degree as would be observed for thrombin. Unlike physiological agonists, STA may not invoke other processes required to augment any mobilization of $[Ca^{2+}]_i$ mediated by the STA-induced formation of $InsP_3$.

4.1.3. STA CAUSES GTPASE ACTIVATION IN HUMAN PLATELET MEMBRANES

In the search for answers as to how STA might mediate these responses, the observation that treatment of platelets with STA and GTPγS simultaneously caused formation of levels of $InsP_3$ higher than did either one of the two suggested that both agents might be acting at the same site and possibly at the G protein level (Fig. 4). On the basis of this, studies were carried out to investigate any stimulatory effect of STA on high affinity GTPase activity in platelet membranes. Results showed that STA caused both time- and concentration-dependent stimulation of high affinity GTPase activity in platelet membranes (Fig. 6). STA-induced GTPase activity plateaued at 10 min, and was

significant for STA concentrations from 0.5 μM to 5.0 μM (Fig. 6). Soon after this work was completed, Kanaho and his group published studies on the effect of STA on the activity of Gi1 that had been purified and reconstituted into phospholipid vesicles (214). They showed that STA activated GTPase activity of reconstituted G_{i1} in a concentration-dependent manner, with a maximal 4- to 5-fold increase at 50-100 μM STA (214). In the present study, we have investigated the effect of STA on the native G protein in the membranes of platelets. The present observations, together with those of Kanaho and others (214) clearly demonstrate that at micromolar concentrations, the PKC inhibitor would activate membrane bound G protein. Other PKC inhibitors, such as H7, and K252a, were not found to induce any significant levels of GTPase activity in platelet membranes (Fig. 6). It seems, therefore, that this effect may be unique to STA.

There is some evidence that PIP_2 hydrolysis in platelets may also be induced by a tyrosine kinase-activated phospholipase C-γ (218). It is unlikely that STA activated this pathway in the current study since STA itself has been found to inhibit tyrosine kinase activity, at nanomolar concentration (74, 218). There are other reported effects of STA, including inhibition and activation of subsets of serine/threonine kinases in platelets, at 1 μM STA (219). Because of these non-PKC specific effects of STA, results of studies on the role of PKC using STA must be interpreted with caution, particularly if the PKC inhibitor was used at micromolar concentrations.

4.1.4. EFFECT OF STA ON PLATELET RESPONSES

STA was found to have no significant effect on platelet secretion (results not shown) or on aggregation (Fig. 7). It appears that at micromolar concentrations, STA both activated and inhibited steps in signal transduction pathways in human platelets. The overall observed effect would therefore depend on the physiological response measured. The lack of effect on platelet secretion or aggregation in our study is likely the result of

the fact that this drug concurrently inhibits PKC. This is largely in keeping with the known synergistic roles played by PKC and Ca^{2+} in such cellular processes. By the use of calcium ionophores or PKC activators such as phorbol esters or membrane-permeable DAGs, it has been established that both $Ins(1,4,5)P_3$ and DAG/PKC are involved in the cascade of reactions that culminates in platelet physiological responses (44, 45, 62, 220). Even though STA may stimulate PLC-induced PIP_2 hydrolysis, the minimal level of $[Ca^{2+}]_i$ mobilized, coupled with its potent inhibitory effect on PKC would be insufficient to induce any physiological responses of platelets, in agreement with published results (4, 89). The contribution of each second messenger arm of the PIP_2/PLC pathway has been assessed only qualitatively and is not precisely known. For example, several groups have shown that the DAG/PKC pathway is prominently involved in the induction of secretion and secondary aggregation rather than with the earlier events of shape change and primary aggregation (61, 208, 221). Results of this study show that 1 to 2 μM STA alone did not cause platelets to aggregate (Fig. 7, traces b, c), neither did it cause any significant secretion in platelets (results not shown). It can be reasoned that at these concentrations of STA, PKC was completely inhibited and that the transient rise in $[Ca^{2+}]_i$ was far below the threshold level needed to trigger platelet aggregation.

On the other hand, PKC inhibition would account for the partial suppression of thrombin-induced, and total suppression of PMA-induced aggregation by STA (Fig. 7, traces c, g). The former agrees with previously published results (4). As implied by present evidence, thrombin-triggered shape change occurred mainly through Ca^{2+}-dependent mechanisms and thus was not blocked by preincubating platelets with STA (Fig. 7 , traces b, c).

4.2. EFFECTS OF MODULATORS OF PKC ON CELL PROLIFERATION

CCRF-CEM (CEM) cells used in these studies were maintained in the logarithmic growth phase. The phorbol ester, PMA, and the PKC inhibitor, STA, both affected

proliferation of CEM cells. The PMA- and STA-induced cell proliferation changes were assessed using two approaches, cell counting by Coulter counter, and determination of number of viable cells by the MTT assay. Both techniques yielded similar results, namely, that the cell proliferation rate was reduced following a 16-h treatment with the phorbol ester; in contrast, STA induced cell death and reduced cell proliferation rate during and following 16-h treatment with the PKC inhibitor (Fig. 8A-C). The presence of 0.1% DMSO (vehicle) in cell growth media did not alter the doubling time of CEM cells. Thus, changes in proliferation of CEM cells in this study could not be attributed to the presence of the vehicle.

Following the 16-h treatment of CEM cells with PMA, the cell doubling time was increased 6.5-fold (doubling time for control cells, 21.5 h); cell death occurred during the 16-h treatment with STA, and thereafter (Fig. 8A). The PMA-induced decrease in number of viable cells (as percent of control) was delayed, occurring only after a 16-h treatment with the phorbol ester (Fig. 8B). In contrast, STA caused a decrease in number of viable cells during the 16-h treatment (Fig. 8C). Since PMA and STA both interact with PKC, the latter is, therefore, implicated in the mechanism underlying the PMA-induced decline in cell proliferation, or the STA-mediated decrease in cell numbers. This implies that PKC activity may be required for cellular proliferation (93, 94, 133, 222-224). During the 16-h treatment with PMA (that is, from T_{-16} to T_0, in Fig. 8A, B), there was no significant decline in cell proliferation even though PKC activity was down-regulated (as observed in Fig. 9, discussed later). Thus, down-regulation of PKC may have preceded PMA-induced reduction in cell proliferation rate. The decline in cell proliferation observed following the 16-h treatment with PMA and that induced by STA may reflect the beginning of PMA- or STA-induced differentiation, since the two PKC modulators have been reported to induce cell differentiation (93, 94, 129, 133, 150, 151, 154, 222-224, also see 225 for review).

Various cell types have been induced to differentiate using phorbol esters such as PMA (TPA). The induction of differentiation of cells in studies following prolonged

treatment with PMA, a condition similar to that used in current studies has been reported. For instance, PMA induced differentiation of K562 cells following 24-h treatment (151), and also of HL-60 cells after 48 h (93), or 72 h of treatment (129). Also observed were induction of differentiation and phenotypic changes in the T-leukemic cell lines RPMI 8402, HPB-MLT cells, and in the T-lymphoblastoid lines, CCRF-CEM, MOLT-4 and MOLT-3 cells, following 2 to 4 days treatment with PMA (94, 133, 222). Similarly, Guerrin and others (223) reported PMA-induced differentiation of human breast cancer cells MCF-7, 6 days after treatment with the phorbol ester. These reports, together with these observations, show that PMA caused a decline in cell proliferation and induced differentiation of proliferating cells, all of which occurred several hours after treatment of cells with phorbol ester, thus supporting the view that down-regulation of PKC is the cause of the changes in cell proliferation and the induction of differentiation.

The idea that persistent activation of PKC resulted in a decline in cell proliferation or induction in differentiation is also supported by the observations of studies using membrane-permeable DAGs. Results of studies using membrane-permeable DAGs, 1-oleoyl-2-acetylglycerol and 1,2,-dioctanoylglycerol, indicated that DAGs could not induce any monocytic differentiation of human myeloid leukemia cells although these agents do activate PKC (see 225 for review, 231). DAGs are incapable of persistently activating PKC since they are rapidly broken down. Therefore, down-regulation of PKC which could not be elicited by the DAGs, may have been responsible for the induction of differentiation of cells in studies where prolonged treatment with phorbol ester has led to decreased cell proliferation or induced differentiation. The onset of PMA-induced differentiation may be characterized by partial or complete block of cell proliferation (224). It was reported that almost complete inhibition of cell proliferation characterized the PMA-induced differentiation of human erythroleukemia, K562 cells by PMA (224).

The low percent of viable PMA-treated cells relative to control (vehicle-treated) cells (observed with either increasing concentration of PMA or increasing duration of

subculture) in Fig. 8B does not, however, suggest PMA cytotoxicity. This observation could be explained on the basis that during the 24-96 h subculture of previously PMA-treated cells, proliferation rate of these cells decreased (Fig. 8A), which, in relation to control (vehicle-treated) cells, would be evident as decreased numbers of viable cells as percent of control (Fig. 8B).

The effects of phorbol esters on cell proliferation and differentiation are varied among different cell types. Notwithstanding the PMA-mediated decrease in cell proliferation in the present study and induction of differentiation observed in the studies of others (129, 94, 222, and references therein), phorbol ester-induced inhibition of differentiation of cells has also been reported including phorbol ester-mediated blockade of differentiation of erythroleukemia (226), neuroblastoma (227), chondroblasts (228), 3T3-preadipocytes (229), and myoblasts (230). This shows that the cell type would influence the outcome of phorbol ester treatment of cells.

Results of the current study also show that STA induced a decline in cell numbers during the 16-h incubation and shortly afterwards (Fig. 8A, C). The decreased cell numbers were more pronounced during the 16-hr incubation than after drug removal. As inhibition of PKC by STA would be achieved in minutes as previous studies suggest (4, 83, 89, 90, 183-185 and see 225 for review), reduction in cell numbers caused by STA during the 16-h incubation implicates PKC in the underlying mechanism. Reduction in cell numbers during the 16-h treatment period and shortly afterward indicates that STA-induced cell death predominated over cell proliferation, but neither of the two processes predominated during the next 20 to 96 h after treatment.

Blockade of cell cycle progression by STA-mediated PKC inhibition may be the basis for the STA-induced changes in CEM cell proliferation rate. STA-induced cell cycle arrest and/or differentiation of human lung epithelial EKVX cells at G1 (150), of human leukemia K562 cells (151), and of nontransformed mammalian cells at G1, or G1 and G2 (152), have been reported. It was also reported by Baltuch and others (223) that altered

PKC activity was the basis for hyperproliferative state of glioma cells, and that STA, at concentrations that inhibit PKC, inhibited glioma proliferation. Also, in murine leukemia A65 cells, another PKC inhibitor, H7, raised the proportion of cells in the G0/G1 phase and decreased the percentage of cells in the S phase, without having much effect on cells in the G2/M phase (233). On the other hand, STA may also inhibit some other protein kinases involved in cell cycle progression to cause changes in the latter and in cell proliferation. For example, it was reported that STA-induced cell cycle arrest at G2, in normal and transformed mammalian cells, was due in part to inhibition of p34cdc2 kinase, a cell cycle kinase (234). The possibility that STA-induced decrease in cell numbers may be by inhibition of PKA or tyrosine kinase is also not ruled out since the PKC inhibitor inhibits these other two protein kinases with comparable IC_{50} values (83).

Individual PKC isozymes may play specific roles in cellular proliferation and in induction of differentiation. For example, prolonged (for 24-48 h) treatment of cells with PMA showed that PKC-β activity was necessary and sufficient for phorbol ester-induced growth arrest and differentiation of HL-60 promyelocytic cells and that the phorbol ester effect on PKC-α and PKC-δ was insufficient for the induction of differentiation (235). Other studies also showed that in human-derived A549 lung and MCF-7 adenocarcinoma cell lines, TPA or bryostatin caused complete down-regulation of PKC-ε, and at 100 times higher concentrations, the PKC activators down-regulated PKC-α with growth arrest (236). In contrast, it was found in another study that PKC-δ, and to a lesser extent, PKC-ε and PKC-ζ play a role in the induction of terminal differentiation of murine erythroleukemia cells (237). Still in other studies, PKC-α, -βII, and -ζ isozymes were observed to be expressed in proliferating human erythroleukemia K562 cells, and the levels of these isozymes were reduced following PMA-induced differentiation (238). These and other observations show that individual PKC isozymes may be responsible for proliferation and differentiation in some cells. The ability of PKC modulators to alter cell proliferation or induce differentiation may thus be affected by the types of isozymes of

PKC present in particular cells. The existence of PKC-β isozyme in CEM cells has been reported (239). It may therefore be reasoned that the PKC-β and any other isozyme present in cells used in the present study are susceptible to modulation by PMA or STA, a modulation which resulted in changes in cell proliferation rate.

4.3. DOWN-REGULATION OF PKC ACTIVITY IN CEM CELLS FOLLOWING TREATMENT WITH PMA

Since observations in the present studies showed that a reduction in cellular abundance of NBMPR binding sites and nucleoside transporter activity followed prolonged treatment of cells with phorbol ester (to be discussed later), it was suggested that phorbol ester-mediated PKC down-regulation was the cause for the decrease in transporter activity. Thus, changes in NT activity following prolonged treatment of cells with phorbol ester could be attributed to the reduced PKC activity. In the literature, persistent activation of PKC by phorbol ester was reported to result in degradation of the kinase by Ca^{2+}-activated proteases, such as calpain (2, 3, 32). For example, prolonged incubation of cells with phorbol esters has been reported to result in low PKC activities (240-243). In one study, exposure of neuronal cells to phorbol ester for 3 h decreased PKC activity by 80-90% of control (205). It was therefore necessary that we assayed the activity of PKC in cells treated with PMA for prolonged periods.

Results from the current study show that following a 16-h treatment of CEM cells with PMA, the activity of PKC was significantly reduced in both cytosolic and membrane fractions (Fig. 9). Total basal PKC activity in control (vehicle-treated) cells of 162 ± 38 pmol per min per mg protein in the present study is less than levels observed in another cultured cells, renal carcinoma SMKT-R3 cells, of 586 ± 145 pmol per min per mg protein (244). Differences in cell types may account for the difference in values observed. Treatment of CEM cells with 100 nM PMA for 16 h caused 96.4% decrease in total PKC

activity in our study which compares favorably with the of 93.7% in total PKC activity caused by treatment of SMKT-R3 cells with 10 nM PMA for 12 h (244). By inference, phorbol ester-PKC down-regulation may be the underlying cause for the decrease in cell proliferation rate that followed the 16 h treatment with PMA, and the decreased cellular abundance of NBMPR binding sites and NT activity (discussed below).

The response of PKC levels to prolonged treatment of cells with PMA may be influenced by the cell type. Thus, decreased PKC activities were observed following 3, 10, 12 or 24 h of treatment with phorbol ester of neuronal cells (205), Swiss-3T3 fibroblasts (240), rat soleus muscle (241), or HepG2 cells (242) respectively. In our study, we have observed that PKC activity was drastically reduced following 16-h treatment of CEM cells with PMA.

4.4. EFFECTS OF PKC MODULATORS AND PROTEIN PHOSPHATASE INHIBITORS ON NUCLEOSIDE TRANSPORTER ACTIVITY AND THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES IN CEM CELLS

4.4.1. THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY IN CEM CELLS TREATED WITH PKC MODULATORS

To gain further insight into the role of PKC in regulation of cellular abundance of NBMPR binding sites and NT activity, and also to clarify the obscurity surrounding this subject, we have conducted studies to assess changes in abundance of NBMPR binding sites and rate of transporter influx following treatment of CEM cells with the PKC

modulators, PMA or STA. CEM cells employed in these studies were maintained in the logarithmic growth phase.

4.4.1.1. EQUILIBRIUM BINDING STUDIES OF NBMPR

The cellular abundance of NBMPR binding sites (B_{max}) and the binding affinity, K_D, in control (vehicle-treated) CEM cells in the current study were $17.4 \pm 0.9 \times 10^4$ molecules/cell and 0.39 nM respectively (Fig. 10A). The reported values of B_{max} and K_D for these cells reported in the literature are 33×10^4 molecules/cell, 0.5 nM (245), and 17.7×10^4 molecules/cell (K_D was not given) (246). The value of B_{max} of NBMPR binding in CEM cells in the current study is similar to literature values (246). The differences in reported B_{max} values might be attributable to the use of different clones of CEM cells in each study.

Incubation of CEM cells in the presence of PMA resulted in time- and concentration-dependent decreases in B_{max} of NBMPR binding without any changes in equilibrium dissociation constant, K_D (Fig. 10A -10D, Tables 1 and 2). Significant changes in B_{max} were observed following longer times, 8 h or more, of incubation of cells with PMA. Since it was observed that in CEM cells treated for 16 h with PMA, PKC activity was drastically reduced (discussed in section 4.3, Fig. 9), lower B_{max} value observed in cells treated for 16 h with PMA is the consequence of PMA-induced PKC down-regulation. The decrease in B_{max} for NBMPR binding is in agreement with the findings of Lee and others (93) who showed that prolonged (48 h) incubation of HL-60 cells with PMA resulted in decline in NBMPR-sensitive nucleoside transport. In that report (93) however, PMA-induced PKC activation was suggested as the basis for the decline, contrary to observations in the literature that prolonged incubation of cells with PMA would down-regulate PKC (2, 3, 6, 29). In spite of this statement, the decline in NBMPR-sensitive nucleoside transporter activity as observed by Lee and others (93) following prolonged treatment of HL-60 cells with PMA agrees with our result. Our

results, however, contrast with the observation that PKC activation by PMA (10 min treatment) resulted in decreased B_{max} of NBMPR binding in chromaffin cells (95). In the current study, conditions that are capable of activating PKC did not affect equilibrium binding of NBMPR (Fig. 16A), while PKC down-regulation resulted in a decrease in number of high affinity NBMPR binding sites. The decline in B_{max} of NBMPR binding may be indicative of the beginning differentiation of PMA-treated cells, since proliferating cells express higher levels of NBMPR binding sites than non-proliferating, differentiated cells (108-113). Moreover, the PMA-induced decrease in B_{max} of NBMPR binding occurred prior to the PMA-mediated decrease in cell proliferation (Fig. 8A, B). This is deduced from the results that significant decrease in the number of high affinity NBMPR binding sites occurred during 8 or 16 h treatment periods (Fig. 10A) in contrast with decreased cell proliferation of PMA-treated cells that was evident only following 16 h treatment period (Fig. 8A).

Our observations and those of others (93, 94) suggest that PKC down-regulation is an event that leads to a decrease in the number of high affinity NBMPR binding sites and NT activity. However, *N,N*-dimethylformamide (DMF), an agent which may or may not interact with PKC, induced a decline in high affinity NBMPR binding sites and NT activity in HL-60 cells (114). Supposing that DMF does not interact with PKC, this would suggest that other mechanism(s) for regulating NT activity may exist. Since N,N-dimethylformamide concurrently induced differentiation of HL-60 cells in the study in question, it may imply that decline in NT activity could occur with induction of differentiation in the absence of PKC modulation if DMF was not capable of modulating PKC activity. For the reason that non-proliferating, differentiated cells have been shown to possess fewer NBMPR binding sites than proliferating, undifferentiated cells (108-113), cells may possess a biological mechanism by which cellular abundance NBMPR binding sites and activity of NT may be reduced prior to or during differentiation.

Since the current study showed that the decrease in NBMPR binding sites in PMA-treated cells was the consequence of PKC down-regulation, it came as no surprise that a short time of incubation (30 min) of CEM cells with phorbol ester would produce no changes in B_{max} of NBMPR binding (Fig. 10A). While PKC is activated during short treatment periods, that is in minutes (225), the protein kinase would not be down-regulated during such intervals. This implies that the duration of exposure of CEM cells to PMA is a critical factor in determining the outcome of the phorbol ester treatment. The observation that a short period of treatment of cells with PMA did not result in any change in equilibrium binding of NBMPR contrasts that of Delicado and others (95). In the latter study, a 10 min treatment of chromaffin cells with 100 nM PMA led to decreased B_{max} of NBMPR binding and NT activity (95). Differences in cell types might be the cause for the differences in these observations.

The cellular abundance of NBMPR binding sites and NT activity have been shown to vary with the cell cycle phase. The number of NBMPR binding sites in cells are positively correlated with the fraction of cells in S-phase (247). For instance, an increase in NT activity was observed when cultures of S1 macrophages synchronously entered S-phase (248). It is unlikely, however, that in our study, PMA decreased the cellular abundance of NBMPR binding sites and NT activity by arresting cells in any particular phase of cell cycle since changes in B_{max} for NBMPR binding and NT activity induced by PMA occurred during the 16-h treatment, prior to changes in cell proliferation rates, which occurred only following 16-h PMA treatment (Figs. 8A and 10A).

Significant decreases in B_{max} of NBMPR binding following treatment of CEM cells with PMA occurred at concentrations of phorbol ester that are relevant to PMA-PKC interaction. The concentration of PMA that caused a 50% decrease in B_{max} of NBMPR binding, IC_{50}, is 9.2 nM (Fig. 10C, D), which is an effective concentration for PMA-PKC interaction (29, 249), thus further supporting the idea that decreases in NT activity and

B_{max} for NBMPR binding observed in PMA-treated CEM cells are associated with PMA-induced PKC down-regulation.

To strengthen the view that PMA-mediated down-regulation of PKC was the cause of the decline in B_{max} of NBMPR binding in current study, we investigated the effect of another PKC-active phorbol ester, phorbol 12, 13-dibutyrate (PDBu), on equilibrium binding of NBMPR in drug-treated CEM cells. Treatment of CEM cells with PDBu for 16 h caused a significant reduction in B_{max} of NBMPR binding without significant change in K_D (Fig. 10E). The similarity of results suggests that as for PMA, PKC down-regulation following the prolonged treatment period with PDBu caused the decrease in B_{max}. In contrast, incubation of CEM cells with 4α-phorbol 12, 13-didecanoate (4α-PDD), a phorbol ester that does not activate PKC (210), did not produce significant changes in B_{max} or K_D (Fig. 10E), also supporting the idea that decreases in NT activity and number of high affinity NBMPR binding as a result of treatment of CEM cells with PMA were PKC-mediated.

In summary, evidence from our study suggests that the phorbol ester, PMA, induces a decrease in B_{max} of NBMPR binding without change in the equilibrium dissociation constant, K_D. No change in B_{max} could be elicited following short treatment period of cells with PMA. Thus, PMA-induced PKC down-regulation may underlie the phorbol ester-mediated decline in cellular abundance of NBMPR binding sites.

Treatment of CEM cells with STA, an inhibitor of PKC activity, caused both time- and concentration-dependent decreases in B_{max} of NBMPR binding sites without any significant changes in K_D (Figs. 11A-11D, Tables 3 and 4). Thus, both PMA and STA exhibited similar effects. This means that PKC down-regulation underlies the PMA-induced decrease in B_{max} of NBMPR binding while inhibition of PKC activity by STA is the cause for the decreased in B_{max} in cells treated with either agent. These observations

are in agreement with those made by others using phorbol ester-induced down-regulation or STA-induced inhibition of PKC to achieve the same purpose (116-118). For example, down-regulation or inhibition of PKC similarly inhibited insulin effects on 2-deoxyglucose uptake (116), caused a significant reduction in α-thrombin-induced formation of phosphatidylethanol in human umbilical vein endothelial cells (117), and diminished IgE-mediated histamine release in skin mast cells (118). Based on such reported findings, as well as ours, it is be said that the effects of PMA or STA, in the current study, are the results of the suppression of PKC activity by the two modulators.

The STA-induced decrease in B_{max} of NBMPR binding may be related to the decrease in cell proliferation and cell numbers mediated by STA (Fig. 8A, C). This would suggest that STA-induced PKC inhibition caused decreases in NT activity and B_{max} for NBMPR binding and proliferating rate of CEM cells. Thus the decreased NT activity and cellular abundance of NBMPR binding sites induced by STA may be an event that occurred during transition from proliferation to differentiation.

The effect of STA on B_{max} of NBMPR binding was evident following treatment of cells with the PKC inhibitor for 6 h or more (Fig. 11A and Table 3) although, in studies of PKC inhibition, the STA mediated inhibition of the protein kinase has been evident in minutes (4, 83, 88-91, 183-185). The observation of the present study that STA-induced decrease in B_{max} of NBMPR binding was detected only following hours of treatment with STA (Fig. 11A and Table 3) may reflect the fact that the measured parameter is distal to PKC inhibition and may be associated with the events that prepare the cell for proliferation changes and induction of differentiation. The implication of this is that, in spite of the rapidity of achieving STA-mediated PKC inhibition, it may take hours for changes subsequent to PKC inhibition to be noticed. For instance, STA-induced changes in cell cycle progression, inhibition of cell proliferation, and induction of differentiation of cells were observed hours after STA-treatment (150-152, 154). Thus the interval between

application of STA and the observation of induction of differentiation may be influenced by the events following PKC inhibition, that is, cell cycle arrests, growth/proliferation inhibition, etc., which may occur downstream to PKC inhibition. In the same manner, changes in NT activity and high affinity NBMPR binding sites could be downstream to PKC inhibition, implying that it would take much longer than it takes for PKC inhibition before any induced changes may be observed. This would make the effect of STA on NT activity time-dependent.

The STA-induced decrease in B_{max} of NBMPR binding was found to be significant at STA concentrations that are capable of inhibiting PKC. For example, following treatment of CEM cells with 10 nM STA for 16 h, B_{max} of NBMPR binding was reduced to 70% of control (vehicle-treated) cells (control B_{max} is 17.4×10^4 molecules/cell, Table 4). This suggests that the STA-mediated decrease in B_{max} of NBMPR binding sites is a consequence of PKC inhibition although it does not rule out the possibility that at 32 nM, STA could have inhibited PKA (IC_{50}, 8 nM), or tyrosine kinase (IC_{50}, 6 nM) (74).

Other PKC inhibitors, GF 109203X (bisindolylmaleimide, BIM) and H7, both altered the equilibrium binding of NBMPR in CEM cells treated with either of these agents (Fig. 11E and Table 5). In CEM cells pretreated with BIM or H7 for 16 h, B_{max} of NBMPR binding sites was reduced to about 81% and 58.2% of control (17.4×10^4 molecules/cell) respectively. BIM, like STA, inhibits PKC at the ATP binding site (211). BIM was reported to be a more specific inhibitor of PKC inhibitor than STA, although is less potent than the latter in inhibiting PKC in intact cells (211). H7 on the other hand is less selective and inhibits other protein kinases as well (211). Nonetheless, using these two inhibitors, we have been able to substantiate the STA-mediated decrease in B_{max} of NBMPR binding sites in CEM cells.

The decrease in B_{max} of NBMPR binding sites observed in BIM-treated CEM cells was smaller compared to that observed in STA-treated cells (compare Figs 11A and 11E). This may be due to lower potency of BIM in inhibiting PKC than STA. It is reported that BIM inhibited isolated PKC isozymes with IC_{50} values of 10 to 20 nM (211). However, in intact cells, BIM inhibited PKC-mediated phosphorylation, with IC_{50} values 10- 20-fold higher than values obtained for STA (211). Similarly, platelet responses such as aggregation, were inhibited by BIM with IC_{50} values also 10 - 20-fold greater than for STA (211). Intracellular concentrations of BIM may not be sufficient to effectively inhibit PKC. This may be because, as a derivative of maleimide, in traversing the plasma membrane, BIM may covalently bind to sulfhydryl groups in the plasma membrane (212, 213) which would decrease the concentration of drug inside cells. Micromolar concentrations of BIM are the suggested concentrations for PKC inhibition studies in intact cells (211).

In preliminary experiments (results not shown) we observed that treatment of CEM cells with 4 μM BIM and higher concentrations resulted in decreased cell numbers. For these reasons, we used 2 μM BIM in the current study. This is in line with the idea of studying an agent that would be beneficial in the use of nucleoside analogs as anticancer drugs. Such a compound should affect NT activity at sub-toxic concentrations. We used a concentration that has a reasonably low cytotoxicity but was unable to significantly lower B_{max} of NBMPR binding. Since higher concentrations caused a dramatic decrease in cell numbers, it became apparent that BIM may not be clinically useful since it may be incapable of producing therapeutic benefits at non-toxic concentrations.

H7, on the other hand, was effective at decreasing B_{max} of NBMPR binding sites in drug-treated cells at a concentration relevant to its inhibition of PKC (Table 5), which supports the idea that STA-induced PKC inhibition was the basis for the decrease in B_{max}

in STA-treated cells. However, this does not rule out the involvement of PKA or tyrosine kinase in the effects observed for either STA and H7.

To support our suggestion that the effect of STA is a consequence of inhibition of PKC, inhibitors specific for other protein kinases but not PKC, namely, HA1004 and genistein, were employed in this study. HA1004 is an analog of H7, and a more specific inhibitor for PKA and cyclic GMP-dependent protein kinase (250, 251), while genistein is a tyrosine kinase inhibitor (150, 251). Treatment of CEM cells for 16 h with either of these agents did not reduce B_{max} of NBMPR binding in drug-treated cells (Table 5). Thus, neither inhibition of PKA, cGMP-dependent protein kinase nor of tyrosine kinase affected B_{max} of NBMPR binding in CEM cells treated with inhibitors of any of these protein kinases. On the basis of these results, we ruled out the involvement of PKA, cGMP-dependent protein kinase or tyrosine kinase in the STA- or H7-mediated decrease in B_{max} of NBMPR binding. The results of this study support the hypothesis that inhibition of PKC causes a decline in B_{max} of NBMPR binding with no change in K_D in CEM cells treated with PKC inhibitors, STA or H7.

4.4.1.2. IS THE TIME-DEPENDENT EFFECTS OF PKC MODULATORS ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY RELATED TO TRANSPORTER TURNOVER?

A nucleoside transport protein turnover cycle as suggested by Torres and others (252), may play a role in the decrease of high affinity NBMPR binding sites and nucleoside transport activity. The NT protein turnover rate in plasma membrane however, is unknown. In the study in question, localized compartments, such as exosomes, which may act as possible carriers of non-functional nucleoside transporters, were observed in

cultures of chromaffin cells (252). This may be cellular machinery to regulate the abundance of NBMPR binding sites and NT activity. NT protein may go through cycles of internalization from membrane, degradation, and subsequent recruitment of new transporter proteins; it may require a long period of time to complete one cycle. This concept seems likely in view of the observation that cytoplasmic pools of some membrane proteins exist, including NBMPR binding sites (253), and CD4 (254). Such observations suggest that a transfer process for specified proteins between membranes and cytoplasmic pools might occur. In support of this concept are the findings of Torres and others (1992) (252) who reported that photolabeling of the nucleoside transporter with [^{3}H]NBMPR, induced a biphasic down-regulation of the transporter protein from the plasma membrane with a first half-life of 2.3 h in chromaffin cells. During this half-life time, the transporter protein was internalized and 50-60% was destroyed while the remainder reappeared in the plasma membrane to undergo a second phase of disappearance cycle with a half-life of 35 h (252). Since NT protein in the plasma membrane of CEM cells may undergo a similar process, that is internalization, degradation and recruitment, the reduction of NBMPR binding by PKC inhibitors, such as STA, may not be evident in minutes, (in spite of its rapid inhibition of PKC) until such a time that the existing transporter proteins have gone through the normal one cycle of internalization and degradation, as suggested by Torres and others (1992) (252). Following this stage, the subsequent recruitment of fresh transporter protein may be impaired because of incompetence of the PKC-dependent biological mechanism required for recruitment. Such a mechanism would become non-functional as a consequence of PKC inhibition. This idea is related to the suggestion (above) that PKC down-regulation induced by PMA would also impair the PKC-dependent recruitment process. It is for this reason that both PMA-induced down-regulation and STA-induced inhibition of PKC would achieve similar results. The effect of STA on cellular abundance of NBMPR binding sites and NT activity thus could be separated into two components: PKC inhibition, and the blockade of PKC-dependent

transduction on the levels of NT activity. While it is unlikely that the first process is time-dependent, the latter is likely to be.

4.4.1.3. RATE OF INFLUX OF ADENOSINE IN CEM CELLS

The observed decreases in cellular abundance of NBMPR binding sites in cells treated with PMA or STA suggested that as the number of high affinity NBMPR binding sites was reduced the transport activity would be decreased. As expected, the inward fluxes of Ado declined simultaneously with the decrease in B_{max} of NBMPR binding following treatment of CEM cells with PKC modulators. Kinetic analysis of Ado influx in CEM cells treated with either PMA or STA indicated that treatment of cells with either of these agents decreased the V_{max} of Ado influx into drug-treated cells without affecting the K_m. These responses correlated with the effects of PKC modulators on B_{max} and K_D of NBMPR binding.

Inward fluxes of Ado in the current study were measured by initial rate methods. This is a crucial requirement for the reason that only initial rates of uptake are measures of the unidirectional inward flux. Procedures to determine initial rates include determining time-courses of permeant uptake and calculation of the slopes of the tangents to the curves at time zero. Another approach to measurement of initial rates is to measure permeant accumulation over a brief time period during which progress curves are linear, and fitting the data by linear regression. In the current study, time courses were linear during the first 9 s of uptake. Progress curves were fitted to data points by linear regression and slopes were a measure of the initial rate. The intracellular accumulation of permeant was calculated taking into consideration permeant that was present within extracellular spaces in cell pellets.

Of the several NT processes that have been described in mammalian cells (102), only *es* is expressed in CEM cells (102). The transporter has a high affinity for and is inhibited by NBMPR, with K_D of less than 1 nM (255-257). In the current study, it was observed that the sensitivity of the transporter to NBMPR remained the same after treatment of cells with PMA or STA and that such treatment did not induce any other NT activity in cells. This is deduced from the observations that fluxes of Ado measured in the presence of NBMPR were very minimal (Figs. 15A-Fig. 17, Fig. 18C, Fig. 18D).

Treatment of CEM cells with PMA caused both time- and concentration-dependent reductions in the rate of influx of Ado into cells (Fig. 12Aii - 12D, Tables 6 and 7). These changes occurred concomitantly with reduction in abundance of NBMPR binding sites. Both types of changes were observed prior to the onset of reduction in cell proliferation that followed 16-h treatment with PMA (discussed in 4.2.1. above). Depending on which transporters are present in cells, NBMPR occupancy of sites on the NT polypeptide may be correlated with inhibition of nucleoside transport (101, 255-257), suggesting that a direct relation may exist between abundance of NBMPR binding sites and *es* nucleoside transport activity. The presence of only NBMPR-sensitive (*es*) nucleoside transport in CEM cells (102), is consistent with the observation in the current study that decreases in B_{max} of NBMPR binding sites following treatment of CEM cells with PMA is correlated with PMA-mediated decreases in Ado influx rate.

Incubation of CEM cells with 100 nM PMA caused a time-dependent reduction in the rate of Ado influx into cells (Fig. 12Aii, Fig. 12C and Table 6), with significant decreases in transport rate following prolonged treatment periods. As with the NBMPR binding studies, PKC down-regulation (Fig. 9) is correlated with the decline in Ado influx rate. In agreement with our results, are the studies of others showing that prolonged treatment of cells with phorbol ester resulted in reductions in nucleoside transport activity (93, 94). Prolonged treatment of cells with phorbol esters in those studies implicates PKC

in the observed decreases in facilitated diffusion of nucleosides. However, in none of those reports was PKC down-regulation identified as the cause for the changes even though the conditions used (24 to 48 h of treatment of cells with PMA) would result in PKC down-regulation. The PKC assay carried out as part of this project (Fig. 9, section 4.2.2), and studies of others (28, 29), show that prolonged exposure of cells to PMA did down-regulate PKC, thus implicating the latter activity in decreases in nucleoside transport activity observed in PMA-treated cells. Thus, the present study clarifies somewhat the conflicting reports that are associated with the role of PKC in the regulation of cellular abundance of NBMPR binding sites and NT activity. Direct PKC activation by PMA (10 min treatment) decreased Ado influx rate in chromaffin cells (95), and Ado influx in these cells was abolished by PKC down-regulation (95). In contrast, conditions that would activate PKC did not have any effect on NT activity in our studies. These differences in results may be due to different cell types.

Significant decreases in the Ado influx rate were caused by the 16-h treatment of cells with 10 nM PMA and higher (Fig. 12C, Table 7). IC_{50} values for the decrease in rate was 11 nM (Fig. 12D), a concentration capable of causing PKC activation and down-regulation. Similarly, treatment of cells with PDBu led to a significant reduction in Ado influx into cells (Fig. 15), further indicating the involvement of PKC. Treatment of cells with 4α-PDD, a phorbol ester that does not activate PKC (210) and is not capable of down-regulating the kinase, did not change the Ado influx rate, confirming that the effects of PMA or PDBu on NT activity occurred through interaction of the phorbol esters with PKC. The observation that reduction in the rate of Ado influx followed prolonged treatment of cells with PDBu also supports the idea that PKC down-regulation is the underlying mechanism for the decrease in Ado influx.

Rates of Ado influx in DMSO (vehicle)-treated cells and in untreated cells were not different from each other (Fig. 12Aii), showing that treatment of cells with the vehicle

alone did not alter NT activity. Also, rates of Ado influx in 0.1% DMSO (vehicle) -treated cells, or in PMA-treated cells, measured in the presence of 1. μM NBMPR were negligible compared to rates measured in the absence of NBMPR (Fig. 12Aii and 12C), which shows that, as expected, NT in CEM cells is virtually totally sensitive to NBMPR (102), and that no change in the sensitivity of the transporter to NBMPR was induced by PMA treatment. This observation suggests that treatment of CEM cells with PMA did not alter the sensitivity of NT to NBMPR, nor did it induce the expression of another type of Ado transport; in contrast is the report that 48-h treatment of HL-60 cells with PMA induced Na^+-dependent uridine influx (93).

As with PMA, treatment of cells with STA caused time- and concentration-dependent decreases in the rate of Ado influx into CEM cells (Fig. 13A-13D, Tables 8 and 9). The effects of STA on the Ado influx rate were apparently a consequence of the inhibition of PKC because 16-h treatments of CEM cells with HA1004 (a PKA and cGMP-dependent protein kinase inhibitor) or genistein, a tyrosine kinase inhibitor, did not lead to significant changes in the rate of Ado influx in CEM cells, while treatment of cells with H7 caused a significant decrease in rate of Ado influx (Fig. 15). Thus, on the basis of these observations, the possibility of STA or H7 mediating effects through inhibition of PKA, tyrosine kinase or cGMP-dependent protein kinase is ruled out. Also, the experiments with H7 support the results obtained with STA, and together suggest that PKC inhibition by STA is the cause for the decrease in number of high affinity NBMPR binding sites and NT activity.

After longer intervals of exposure of CEM cells to STA, the change in rate of Ado influx induced by STA was increased (Fig. 13A, B and Table 8). After 8 h of exposure, rates of Ado influx were reduced to about 50% of rate in DMSO (vehicle) -treated cells, (0.31 pmol per s per 10^6 cells) (Fig. 13A, Table 8). As discussed above, the time-dependency of the STA effect could be due to the fact that the measured parameter is not

PKC inhibition, but rather it is distal to the inhibition of PKC, and may involve NT protein turnover. Reduction of the Ado influx rate in CEM cells following treatment of cells with STA for 16 h, also showed dose-dependency (Fig. 13C, Table 9).

Some observations have indicated that simultaneous treatment of HL-60 cells with PMA and STA abolished the reductions of facilitated diffusion rates induced by 48-h treatment of cells with PMA (93). This is in contrast to our observation that the simultaneous presence of PMA and STA did not abolish the PMA- or STA-induced decrease in Ado influx rate (results not shown). While the observation of Lee and others (93) is contradictory to current understanding in the literature, based on these ideas, our observation is consistent with such deductions. In theory, PKC down-regulation and inhibition would eliminate PKC activity and therefore should produce similar results. Furthermore it is unlikely that STA or PMA together could mutually prevent effects of these agents, if the effect of STA or PMA is the result of elimination of PKC activity, either by inhibition or down-regulation. Our observation also conflicts with that of Delicado and others (95) who found that down-regulation of PKC produced no effect on NT activity, while treatment with STA caused increase in B_{max} of NBMPR binding in chromaffin cells. Results of our studies, although contrasting with the observations of others, are consistent in the sense that PMA-induced down-regulation of PKC and achieved the same effect as STA.

Effects of treatments of cells with PMA or STA on the kinetic constants of Ado influx were determined with the aim of determining whether changes in B_{max} for NBMPR binding correlated with changes in maximum rate of influx, V_{max}, of Ado influx in PMA- or STA-treated cells. In cells treated for 16 h with either PMA or STA, these agents altered V_{max} without altering K_m for Ado influx in CEM cells (Figs. 14A and 14B), suggesting that under these conditions, the number of transporters decreased in parallel with decreases in NBMPR binding sites (Figs. 10A - 11D).

Results of present study are similar to the findings that phorbol ester-induced PKC down-regulation decreased glucose transporter activity (259). Our observations are only partly in agreement with those of Delicado and others (95) who showed concurrent decreases in B_{max} of NBMPR binding and in V_{max} of nucleoside transport in PMA-treated chromaffin cells. However, their observations were made in cells treated with PMA for 10 min, suggesting that PKC activation was the basis of the effects. Down-regulation of PKC activity, in cells in their study, had no effect on NT activity (95).

In summary, there was a decreased maximal rate, V_{max}, of influx of Ado into cells with no significant change in K_m, following prolonged treatment of cells with phorbol ester or PKC inhibitor. Together with observations made from binding studies, the results suggest that decreased V_{max} is the result of decreased expression (lower B_{max}) of functional NT polypeptides, and that no alteration occurred in the affinity of transporter polypeptides for the permeant or the transporter inhibitor, NBMPR.

It has been discussed above that, following prolonged treatment of CEM cells with phorbol ester, cell proliferation rate declined as evidenced by decreased slope of the growth curve (Fig. 8A, 0 to 96 h), and also that there was decreased cellular abundance (B_{max}) of NBMPR binding sites and reduced rate of influx of Ado. Reports have attributed changes such as phorbol ester-induced reduction in NT activity, inhibition of cell cycle progression, inhibition of cell proliferation, and induction of differentiation, to PKC activation even though prolonged treatment periods (24 h or more) were used (93, 94, 133, and see 225 for review). One reason for conducting this study was to address this issue of PKC activity following prolonged treatment with phorbol ester. This would provide some evidence to support one or the other claims, as to whether PKC activation or down-regulation is the cause for the PMA-induced decrease in cell proliferation, NT activity and the number of high affinity NBMPR binding sites. Overall, the evidence favors the idea that the decreased B_{max} of NBMPR binding sites and decreased rates of Ado

influx following prolonged treatment of CEM cells with phorbol esters is a result of down-regulation or the inhibition of PKC.

4.4.1.4. EFFECT OF "PKC ACTIVATION" ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY IN CEM CELLS

In view of the result that phorbol ester-mediated PKC down-regulation or PKC inhibition resulted in decreases in B_{max} for NBMPR binding, as well as Ado influx rates, studies were carried out to examine any variations in number of high affinity NBMPR binding sites and NT activity in CEM cells following short periods of PMA treatment. The rationale was to stimulate PKC activity and determine the influence of this on NT B_{max} for NBMPR binding and Ado influx rate.

Treatment of CEM cells with 100 nM PMA for 10 min, conditions that would be expected to activate PKC (225), did not produce significant changes in B_{max} of NBMPR binding, K_D (Fig. 16A), or in the inward flux of Ado (Fig. 16Bi and ii). The lack of significant changes in B_{max} for NBMPR binding or Ado influx rate following exposure of cells to PMA for 10 min was interpreted to mean that cellular abundance of NBMPR binding and NT activity were not affected by an increase in PKC activity over this time period. This contrasts with the reports that treatment of chromaffin cells with PMA for 10 min decreased B_{max} of NBMPR binding (95), and that PMA-induced PKC activation led to stimulation of glucose uptake in human erythrocytes (92).

On the basis of this observation, we propose that in the normally proliferating CEM cell, PKC activity is not rate-limiting in the expression of NT which is at its highest

level; that transporter activity could not exceed this level in cells (at basal PKC activity). NT expression may be rate-limited at the level of polypeptide synthesis, so that an increase in PKC activity alone would not up-regulate transporter expression. If this is the case, then activation of PKC may not lead to an increase in NBMPR binding sites or in transport rate. In contrast, if PKC was down-regulated, this would remove PKC-mediated events necessary to maintain the maximal expression of NT, resulting in an overall decrease in B_{max} of NBMPR binding and rate of Ado influx.

Considering the possibility that NT activity may go through a turnover cycle, of degradation and subsequent recruitment of new transporter proteins into the membrane as suggested by Torres and others (252), there are chances that the period of PKC activation, which occurs in minutes (225), may occur several hours before the recruitment of new transporter proteins into the membrane, or before the exposure of transporter binding sites which hitherto may be cryptic in the membrane. As a result, it may be unlikely that measurements of NBMPR binding and Ado influx rates would show any increase, at 5-10 min following phorbol ester treatment of cells. We probed this concept further by conducting experiments that allowed 2 h following 10 min treatment of cells with PMA. Results (not shown) did not show any enhancement in rate of Ado influx into cells, therefore ruling out the idea that the duration of time following PKC activation may not have been adequate to allow activated PKC-mediated process to produce any effect.

4.4.1.5. DOES SHORT TREATMENT WITH PMA REVERSE THE STA-REDUCTION OF NT ACTIVITY?

It seems that the activation of PKC following STA-induced PKC inhibition in CEM cells could not reverse the decrease in NT activity caused by STA. When CEM cells

were pretreated with STA for 16 h, prior to treatment with PMA for 10 min, rate of Ado influx remained low, indicating no reversal of STA-induced decrease in Ado influx rate (Fig. 17). The rationale for the 2-h resting period following 10 min PMA-treatment of STA-treated cells was to allow some time for any possible initiation of events by PMA-induced PKC activation, the events which may be necessary to effect reversal of STA-induced effects. The 2-h period was an estimate only and it is possible that the resting period after PMA-treatment may not be adequate for allowing activated PKC-mediated changes to reverse STA-induced decrease in rate of Ado influx.

Events such as recruitment of new transporter proteins into the membrane, or exposure of binding sites which hitherto have been cryptic and could be accessible only after PKC-induced phosphorylation, may be critical for reversal of STA-induced decrease in rate of Ado influx. The concept of transporter recruitment is in line with the observations and suggestions of Torres and others (252), that nucleoside transporter protein may undergo a turnover cycle of internalization, degradation, and recruitment of new transporter proteins into the membrane. In such a situation, if measurements of NT activity and number of high affinity NBMPR binding sites were carried out earlier than the time required for one complete turnover cycle, one might not observe return of NT activity to basal levels, even though PKC activity may have been restored.

The persistence of STA-induced changes in NT could be viewed as related to STA-induced changes in cell proliferation, or differentiation, a view which would also be in line with an observation, that treatment of cells with STA induced long-lasting changes in cell proliferation and cell numbers (see Fig. 8A). Since the decreased NT activity and the number of high affinity NBMPR binding sites induced by STA was long-lasting, it is suggestive that the STA-induced decline in cell proliferation may be related to the STA-induced decrease in NT expression. The implication of this is that reversal of the reduced NT activity and low numbers of high affinity NBMPR binding sites would not be evident

until after several days, should it be associated with the processes related to reversal of proliferation rate to normal.

4.4.2. EFFECTS OF PROTEIN PHOSPHATASE INHIBITORS ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY IN CEM CELLS

Results of the current project show that conditions that activate PKC above basal levels did not increase cellular abundance of NBMPR binding sites and NT activity, while elimination of the kinase activity led to decrease in B_{max} for NBMPR binding and NT activity. On the basis of these observations, we propose a model for the role of PKC in *es* NT expression. In this model, the nucleoside transporter polypeptide is perceived to be phosphorylated in the membrane of the CEM cells. There is absolute requirement for basal PKC activity in maintaining the expression of NT. Activation of PKC does not cause an increase in transporter activity or number of high affinity NBMPR binding sites. The model predicts that PKC-mediated phosphorylation of nucleoside transporter polypeptides, or of some intermediate membrane protein, is necessary for NT activity. However, elimination of PKC activity, either by phorbol ester-induced PKC down-regulation, or by inhibition of PKC, removes the required PKC-induced phosphorylation steps for maintenance of NT expression.

The general pattern among proteins that require phosphorylation for functioning is that they may also undergo dephosphorylation. It was seen as a plausible idea to exploit any possible dephosphorylation steps that transporter polypeptides may undergo. For instance, if NT expression required PKC-mediated phosphorylation and that elimination of PKC activity caused a decrease in expression of NT, it may be possible to circumvent that

decrease by blocking the dephosphorylation step. This is because blockade of dephosphorylation would maintain the normal level of phosphorylated nucleoside transporter polypeptides even though PKC activity may have been inhibited. To test this hypothesis, we have investigated the effects of protein phosphatase inhibitors, okadaic acid (OKA) and calyculin A (CLA), on equilibrium binding of NBMPR and Ado influx rates in CEM cells. OKA and CLA are inhibitors of PP1 and PP2A (175-179).

In cells treated with OKA and CLA, no changes were observed in the B_{max} of NBMPR binding, or in the inward fluxes of Ado (Fig. 18A-D). This observation is in agreement with the proposed model, which predicts that phosphorylation of NT polypeptides is complete under conditions of resting PKC activity; if this model is correct, a decrease in dephosphorylation would not result in enhancement of NT activity or increase in number of high affinity NBMPR binding sites. This observation is similar to the results obtained for short treatment of cells with PMA (which would cause PKC activation), and supports the proposed model.

A key finding was that OKA or CLA blocked the STA-induced decrease in B_{max} of NBMPR binding (Fig. 18A and B). To explain this result, we would have to view the expression of NT as dependent on a cycle of phosphorylation and dephosphorylation, as suggested in the proposed model. Blockade of PKC by STA would result in a deficiency in the phosphorylation step, while dephosphorylation step by protein phosphatases continued. This would ultimately lead to the decreased expression of NT in the presence of STA, as observed throughout this project. In the presence of protein phosphatase inhibitors, OKA or CLA, however, dephosphorylation step was disengaged at the same time that PKC-mediated phosphorylation was blocked by STA, with the result that the number of high affinity NBMPR binding sites in the membrane did not decrease.

Treatment of cells with STA in the presence of CLA also prevented the STA-induced decrease in Ado influx (Fig. 18D), although simultaneous incubation of CEM cells with STA and OKA did not (Fig. 18C). This anomaly may be due to the fact that OKA is more potent against PP2A than PP1 (175-179) while CLA inhibits both phosphatases with equal potencies (177, 179). This may also suggest that PP1, which is poorly inhibited by OKA, may play a greater role in NT polypeptides dephosphorylation than PP2A. If this was the case, then inhibition of PP2A alone may not be sufficient to cause complete blockade of dephosphorylation of transporter polypeptides in which case, complete reversal of STA-induced decreases in B_{max} of NBMPR binding or Ado influx rate may not be achieved.

The idea that the nucleoside transporter protein, and perhaps other transporter proteins, may be phosphorylated has been proposed by Delicado and others (95), who suggested that PKC-mediated phosphorylation might have caused the decrease in B_{max} of NBMPR binding and of nucleoside transport activity. Also in line with this idea are the reports that activation of PKC increased the phosphorylation of glucose transporters and caused increased glucose uptake in erythrocytes (92), and that PKC-mediated modification of Na^+-independent glucose transport activity occurred in quiescent mouse fibroblasts (98). These studies, as well as ours, provide some evidence that phosphorylation mediated by PKC is required for expression of membrane transporters. From our study, we can say that protein dephosphorylation plays a role in the activity of the *es* transporter in CEM cells

Quite recently, the amino acid sequence of a Na^+-dependent nucleoside transporter, *cit*, from rat jejunum was deduced, and potential PKC-dependent phosphorylation sites were identified (259). The presence of PKC-dependent phosphorylation sites on the nucleoside transporter polypeptides in CEM cells is shown in

these studies; it would be interesting to determine if probes for the *cit* transporter would interact with the *es* transporter.

It could be reasoned that phosphorylation serves as a positive modulation step for NT expression, in contrast to the dephosphorylation step which may serve to decrease NT expression. In that regard, the two contrasting modulatory processes may be a part of the biological cycle of recruitment (and/or expression), removal of transporter from the accessible position in the membrane (or internalization), followed by degradation, and then back to recruitment of new transporters, as suggested by the studies of Torres and others (252). Alternatively, the dephosphorylation step may bring about a decrease in NT expression by causing the dephosphorylated NT to undergo change in conformation which would result in concealment or occlusion of the high affinity NBMPR binding sites. This could result in a decrease in the cellular abundance of accessible NBMPR binding sites, and a decrease in transporter activity.

In summary, use of protein phosphatase inhibitors like OKA or CLA, which block dephosphorylation steps, have shown that attempts to further elevate phosphorylated NT polypeptides or some other protein that is involved in the regulation of NT expression, did not result in increased NT activity or in the cellular abundance of NBMPR binding sites. This would support the proposed model in which, at basal PKC activity, the relevant membrane polypeptides were already phosphorylated. The results also support the view that NT polypeptides (or some other protein) undergoes phosphorylation and dephosphorylation, as the two opposite steps of positive and negative modulation. Blockade of PKC-mediated phosphorylation (by PKC down-regulation or inhibition) would result in lower levels of phosphorylated NT protein, and a consequent decrease in NBMPR binding sites, as well as nucleoside transport rates. This could be blocked by simultaneous treatment of cells with protein phosphatase inhibitors, such as CLA. The protein phosphatase inhibitors act to block the negative modulation step of reducing levels

of phosphorylated NT and thus serves as alternate route to maintain levels of phosphorylated NT, by preventing dephosphorylation steps.

5. SUMMARY AND CONCLUSIONS

5.1. The effect of STA on inositol phosphate signal transduction was studied in human platelets. The following are the observations:

1. STA at micromolar concentrations caused formation of significant levels of $InsP_3$ in both intact and permeabilized human platelets; this phenomenon was inhibited by neomycin, a PLC inhibitor, in intact platelets
2. The STA-induced mobilization of cytosolic calcium was rather weak although the PKC inhibitor did potentiate the effect of thrombin.
3. STA also induced the formation of low levels of $Ins(1,4,5)P_3$, the $[Ca^{2+}]_i$-mobilizing second messenger.
4. STA did not induce any appreciable effect on platelet aggregation or on platelet secretion (results not shown).
5. Micromolar concentrations of STA stimulated GTPase activity in platelet membranes suggesting that the PKC inhibitor activated the receptor-coupled G protein, in agreement with the findings of Kanaho and others (214).
6. Therefore, at micromolar concentrations, STA would not only inhibit PKC, but also invoke non-specific effects such as the hydrolysis of PIP_2, similar to a Ca^{2+}- mobilizing agonist.

5.2. Studies of the effects of PMA and STA on proliferation of undifferentiated T-lymphoblastoid CCRF-CEM (CEM) cells showed that both PKC modulators reduced proliferation of CEM cells. The effects of PMA and STA were attributed to down-

regulation and inhibition of PKC, respectively. Using PKC modulators and protein phosphatases inhibitors we have also investigated the role of protein kinase C in the expression of NT in CEM cells. The following are our findings:

1. Both STA and PMA caused concentration- and time-dependent decreases of rate of Ado influx, and of B_{max} of NBMPR binding in CEM cells without any significant change in the K_D, following incubation of cells with these agents.
2. Significant changes in B_{max} and Ado influx rate occurred only after prolonged treatments periods with PMA or STA, suggesting that PMA-induced PKC down-regulation, or PKC inhibition by STA, was the underlying mechanism for the observed decreases in number of high affinity NBMPR binding sites and NT activity.
3. Prolonged treatment of cells with PMA or STA significantly reduced V_{max} of Ado influx without appreciable changes in K_m; this correlates with the corresponding drug-induced reduction in B_{max} of NBMPR binding sites with no change in K_D.
4. Treatments of cells with H7, another inhibitor of PKC, or PDBu, led to decreases in rate of Ado influx and B_{max} of NBMPR binding without significant change in K_D.
5. The exposure of cells to genistein, an inhibitor of tyrosine kinase, HA1004, a PKA and cGMP-dependent kinase inhibitor, and 4α-PDD, an inactive phorbol ester, did not result in decline in rate of Ado influx or B_{max} for NBMPR binding.
6. The decreased cellular abundance of NBMPR binding sites and NT activity induced by PMA or STA persisted during a 3-4- day period of subculture of cells following withdrawal of agent.

7. Treatment of cells with PMA for 5 or 10 min, conditions known to activate PKC, did not change in NT activity.

8. Treatment of cells with the protein phosphatase inhibitors, OKA or CLA, did not alter the number of high affinity NBMPR binding sites and NT activity; however, OKA and CLA partially or completely blocked STA-induced decline in B_{max} for NBMPR binding respectively; CLA also completely prevented STA-mediated decrease in rate of Ado influx.

9. Results of this study are consistent with the hypothesis that protein phosphorylation by PKC, and dephosphorylation by protein phosphatases, are involved in the regulation of the number of high affinity NBMPR binding sites and NT activity.

6. FUTURE DIRECTIONS

The subject of STA and G protein function is fairly new, with not much information in the literature. The first report that STA may alter G protein activity was made by Kanaho and others (214), around the same period that the present work was carried out. Thus, more exploration of the STA-G protein phenomenon is needed to understand the nature of the interaction between STA and the G protein. Also the types and the fate of the $InsP_3$ that are produced in response to STA treatment need to be identified. It is known that agonist-induced $InsP_3$ undergoes further metabolism with the generation of inositol which is fed back into the inositol pool.

On NT expression and PKC activity, there is also very little information in the literature. Reported findings also show inconsistencies. Unlike phorbol ester- or STA-induced cell growth arrest and differentiation, which has been extensively studied to provide ample evidence for the role of PKC in these processes, PKC and NT expression requires further work. PKC-dependent phosphorylation of NT polypeptides or some other

protein which may be associated with expression of NT remains to be directly proven, although in our study we have used an indirect approach in the form of protein phosphatase inhibitors. Dephosphorylation of the phosphorylated NT polypeptides or the putative phosphoprotein by a protein phosphatase also requires a further direct investigation.

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